AMPA Glutamate Receptor Trafficking in Models of Disease

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Dedication

This work is dedicated to my grandmothers, Dr. Janet Forsythe Fishburn and Mildred Marie Miller. Both are strong, independent women. They have worked hard to foster my love of education from a young age and have set shining examples of the heights that learning can take you to.

Abstract

The signaling between neurons in the brain underlies many crucial processes – from the beating of the heart to remembering that you have a meeting at noon. Indeed, it is proposed that changes in the way neurons communicate with each other form the basis of learning and memory. In this dissertation I will explore the ways in which neurological diseases can affect these integral neuronal functions and explain the changes on a cellular and molecular level. Deficits in AMPAR signaling are found in numerous diseases. I will explore the mechanisms of these deficits using *in vitro* models of three diseases: opioid-related cognitive deficits and addiction, Alzheimer's disease, and Parkinson's disease-related dementia. Background information is presented in the first chapter. In the second chapter the signaling pathways underlying morphine-induced synaptic deficits are delineated. I found that calcineurin is necessary for both functional and structural deficits in AMPAR signaling, while CaMKII is necessary for only the structural deficits. The role of tau in synaptic deficits cause by soluble Aβ oligomers, which are found at elevated levels in Alzheimer's disease patients, are probed in the third chapter. I found that treatment with soluble Aβ oligomers leads to phosphorylation-dependent mislocalization of tau to dendritic spines. Furthermore, treatment with soluble Aβ oligomers leads to decreases in AMPAR signaling that require calcineurin activity and GluR1 residue S845, much like the mechanisms of AMPAR internalization in neurons treated with morphine. The fourth chapter unveils a novel role of tau and GSK3 in synaptic deficits found in neurons expressing A53T α-synuclein. In fact, I discovered that tau is involved in AMPAR signaling deficits found in neurons expressing A53T α-synuclein. Furthermore, both mislocalization of tau and synaptic deficits require phosphorylation of tau by GSK3. This dissertation shows that divergent pathways mediate structural and functional plasticity found in neurons exposed to morphine. Also, I show that deficits in AMPAR signaling in both Alzheimer's disease and Parkinson's disease involve tau mislocalization. These findings shed new light on the signaling pathways involved in AMPAR signaling deficits found in neurological diseases and provide new therapeutic targets for pharmacological interventions.

Table of Contents

List of Figures

Chapter 1: Introduction

I. Synaptic Plasticity

Plasticity refers to changes in communication between cells. It offers an explanation of how learning occurs, how the brain stores memories, and how external stimuli can change signaling properties in the brain. Hebb's postulate was first posited in 1949; it holds that concurrent activation of two cells, or a group of cells, will lead to a greater association between the two cells in that the firing cell will activate the receiving cell at increased levels in proceeding stimulations (Hebb, 1949). The first evidence in favor of this theory was the discovery of long term potentiation (LTP), a cellular model demonstrating an increased signaling connection between cells after a flurry of activity, as proposed in Hebb's postulate (Bliss and Lomo, 1973). Long term depression (LTD), in which low-level activity causes a decrease in the signaling connection between cells, was later demonstrated – this showed that plasticity is not a unidirectional phenomenon (Dudek and Bear, 1992; Mulkey and Malenka, 1992). Along with changes in electrical signaling at synapses, further structural changes in dendritic spines have been identified after induction of LTP and LTD (Matsuzaki et al., 2004; Zhou et al., 2004). Dendritic spines protrude from the dendrites of neurons and are the main postsynaptic site of excitatory glutamatergic signaling in the brain (Cingolani and Goda, 2008; Patterson and Yasuda, 2011). Dendritic spines have long thin necks that lead to a bulbous head – this morphology, along with the binding properties of anchoring proteins found in spines, allow for compartmentalized ionic and protein signaling to occur in individual spines (Bloodgood and Sabatini, 2005; Byrne et al., 2011). Many of the changes in signaling at

the synapse can be mediated by localized signaling events that occur in the postsynaptic dendritic spine or presynaptic button. Over the last 30 years, a number of intracellular signaling proteins have been implicated in LTP and LTD (Cingolani and Goda, 2008; Malenka and Bear, 2004; Nicoll and Roche, 2013; Patterson and Yasuda, 2011). These cellular phenomena are hypothesized to underlie changes in behavior (Kessels et al., 2013).

As understanding of these cellular events deepened, researchers began looking to the synapse to understand diseases. In a variety of diseases changes in signaling between neurons have been described (Kauer and Malenka, 2007; Selkoe, 2002; Sheng et al., 2013). This plasticity can be functional or structural. Specific examples of functional plasticity include changes in AMPA glutamate receptor (AMPAR) currents, changes in the expression of AMPARs at the synapse, and changes in presynaptic vesicle release. Specific examples of structural plasticity include changes in the dendritic spine density and size/shape of spines. In the following chapters, we will investigate plasticity and unravel the underlying intracellular signaling pathways in three different disease models.

II. The Role of Hippocampal μ-Opioid Receptors in Addiction

The role of functional and structural plasticity of dendritic spines in drug addiction has recently attracted great attention from both basic researchers and clinicians. Although it is known that drugs of abuse cause synaptic plasticity, the intracellular signaling pathways through which these changes are mediated are poorly understood.

Greater understanding of intracellular signaling pathway underlying addiction could provide new therapeutic targets for the prevention and treatment of drug addiction.

A. The hippocampus is involved in addiction

The hippocampus is an area of the brain that is involved in learning and memory, particularly spatial memory. Addicts learn to associate drugs with environmental cues; it is believed that the hippocampus mediates this association. Numerous studies have shown that the hippocampus is involved in the addictive process. Intra-hippocampal injection of morphine has been shown to produce conditioned place preference, a behavioral measure of the association of a drug with a drug-taking environment (Corrigall and Linseman, 1988). Morphine exposure modulates gene expression in the hippocampus (Marie-Claire et al., 2007). Pharmacological lesion of the hippocampus has been shown to disrupt the acquisition of cocaine conditioned place preference (Meyers et al., 2006). Injection of methamphetamine into the hippocampus produces both conditioned place preference and drug-seeking behavior (Ricoy and Martinez, 2009). These studies and others suggest an important role of the hippocampus in addiction.

B. Mu-opioid receptors are involved in morphine addiction

Morphine is a pure opioid agonist. It is highly active at μ-opioid receptors (MORs), while less active at κ- and Δ-opioid receptors. Naloxone is an opioid antagonist that mainly blocks MORs; c-Top is an opioid antagonist that blocks only mu-opioid receptors. The positive reinforcing effects of morphine are thought to be mediated by its

actions on MORs in the mesolimbic dopamine pathway. Morphine disinhibits dopaminergic neurons in the ventral tegmental area by binding to MORs and thus inhibiting GABAerigic neurons in the nucleus accumbens (Julien et al., 2008). While this is one pathway through which morphine is thought to cause dependence, a dopamine-independent mechanism has also been suggested (Herz, 1998).

Mounting evidence implicates MORs throughout the brain in the development of morphine dependence. In rats self-administering heroin, high doses of an injected MOR antagonist caused extinction of self-administration (Negus et al., 1993). Under normal circumstances rats injected with morphine develop conditioned place preference and locomotor sensitization (increased locomotor movement upon successive drug treatments), both indicative of the development of dependence. MOR knockout mice do not develop condition place preference or locomotor sensitization in response to morphine (Becker et al., 2000; Matthes et al., 1996). The above studies suggest that MORs are necessary for the development of morphine dependence.

C. Mu-opioid receptors modulate dendritic spine dynamics and AMPAR signaling

Dendritic spines are the site of most excitatory synaptic transmission in the brain. Recent studies have shown that MORs play a critical role in modulating the structure of dendritic spines. The over-activation of MORs by morphine causes a drastic change in the morphology of excitatory synapses; in the hippocampus spine density is decreased by *in vivo* morphine exposure (Robinson and Kolb, 2004). Our subsequent work has shown that chronic treatment of cultured hippocampal neurons with morphine causes not only

spine loss, but functional deficits as well. Morphine inhibits AMPA glutamate receptor function, as measured by decreases in both amplitude and frequency of miniature excitatory postsynaptic currents (mEPSC). These effects were shown to be mediated by postsynaptically-localized MORs (Liao et al., 2007a; 2007b; 2005). Determining the intracellular mechanisms through which morphine modulates structural and functional plasticity will shed new light on the development of addiction. In chapter 2 of this dissertation we will discuss our research into the mechanisms of morphine-induced plasticity.

III. The Synaptic Role of Tau in Alzheimer's Disease and other Neurodegenerative Disorders

Hippocampal plasticity is not unique to models of addiction; recent evidence implicates plasticity in various forms of dementia. Spine loss and deficits in AMPAR signaling are found in hippocampal neurons treated with morphine; similar changes are found in models of dementia. Delineating the molecular mechanisms of disease could pave the way for new therapeutic treatments. In the third and fourth chapters of this dissertation we will discuss possible signaling mechanisms underlying plasticity during early stages of dementia. The commonalities between plasticity found in cultured hippocampal neurons exposed to morphine, soluble Aβ oligomers, and A53T α-synuclein will become apparent as this dissertation unfolds.

Alzheimer's disease (AD) was first described by Alois Alzheimer a century ago (Alzheimer, 1907). The primary symptom of Alzheimer's disease is progressive memory loss accompanied by the inability to form new memories at later stages in the disease. Moreover, the disease can affect decision-making, personality, and other aspects of cognition. AD is estimated to be responsible for 50-60% of dementia cases (Blennow et al., 2006). Dementia is only found in 1% of 60-64 year old individuals; however, among those who are over 85 years of age the rate of dementia jumps to 24-33% (Ferri et al., 2005). In the United States 5.2 million people live with the AD today and more than 25 million are affected worldwide (Alzheimer's Association, www.alz.org). Due to expected increases in life expectancy, this number could reach as high as 81 million by 2040 (Ferri et al., 2005). Although cures for AD and related dementias have not yet been identified our understanding of the disease process has expanded greatly since the discovery of the disease.

A. Plaques and tangles

In his initial 1907 description of AD, Alois Alzheimer described an unusual neuropathology in his demented patient. The diseased brain contained miliary bodies (now known as neuritic plaques) and fibrils (now known as neurofibrillary tangles) which are now considered the pathological hallmarks of AD; this pathology was initially thought to be responsible for neurotoxicity in AD patients (Alzheimer, 1907; Blennow et al., 2006). These lesions are localized to the medial temporal lobe and the cortex, areas involved in memory and cognition (Blennow et al., 2006). Nearly three decades ago,

amyloid β-peptide (Aβ) was discovered to be the main component of neuritic plaques (Masters et al., 1985). Originally researchers did not know the physiological role of Aβ, but eventually found that it is a product of normal cell metabolism; proteolytic cleavage of the amyloid-β precursor protein (APP) produces Aβ (Haass et al., 1992). The amyloid-beta cascade hypothesis posits that dementia is caused directly or indirectly by accumulation of Aβ due to abnormal decreased clearance or increased production of the peptide. In humans with AD mutations in APP or presenilin, affecting production of Aβ, or APOE, affecting clearance of Aβ, have been correlated with cognitive deficits (Blennow et al., 2006). Soon after Aβ was found to be the primary component of plaques, the microtubule-associated protein tau, in a hyperphosphorylated state, was discovered to be the chief component of neurofibrillary tangles (Grundke-Iqbal et al., 1986; Nukina and Ihara, 1986). Tau protein binds to microtubules and plays a role in cytoskeletal development and regulation in neurons; the protein's phosphorylation state is controlled by kinases and phosphates such as glycogen synthase kinase-3 (GSK3β) and protein phosphatase 1 (PP1) respectively (Avila et al., 2004; Buée et al., 2000).

Pathology outside of neuritic plaques and neurofibrillary tangles, e.g. Lewy bodies and vascular infractions, has been found in many patients with clinical AD diagnoses – it is estimated that between 1/3-1/2 of AD patients have a mixed pathology (Kovacs et al., 2008). Furthermore, patients with mixed pathology have a much higher chance of experiencing dementia than those who only exhibit pathology consistent with only one disease (Fotuhi et al., 2009; Langa et al., 2004; Schneider, 2009).

In AD the number of neurofibrillary tangles, but not neuritic plaques, have been positively correlated with the severity of dementia (Arriagada et al., 1992). Interestingly, alterations in tau are found in a number of other neurodegenerative diseases. Tau inclusions and hyperphosphorylation are also found in frontotemporal dementia, corticobasal degeneration, Pick's disease, and other dementias (Avila et al., 2004; Buée et al., 2000). Tau has also been implicated in Parkinson's disease; many patients experience dementia in addition to motor disturbances (Aarsland et al., 2008; Reichmann et al., 2009; Svenningsson et al., 2012). Parkinson's disease is characterized by Lewy bodies, which are primarily composed of a-synuclein, but also contain hyperphosphorylated tau (Ishizawa et al., 2003). The role of tau in a number of neurodegenerative disorders suggests the importance of the protein in the development of dementia. We will discuss the evidence supporting the role of tau in AD below, but first we will explore the evidence that has shifted AD research away from plaques and cell death towards soluble oligomers and synaptic loss.

B. Shifting Winds: Plaques and tangles give way to soluble Aβ and tau

Initial research into AD focused on the neuritic plaques and neurofibrillary tangles found at later time points in the disease. Much has been learned about these hallmarks of AD pathology, but the focus of the research community has shifted towards understanding synaptic deficits that are found at earlier time points in the development of the disease (Ashe and Zahs, 2010; Selkoe, 2002). This is due to findings that have indicated that AD has a long preclinical phase preceding cell death and brought the

relationship between plaques, tangles, and neurotoxicity into question. Aβ plaques are found in 40% of elderly adults who have not been clinically diagnosed with dementia (Hulette et al., 1998). Subsequent inquiry has confirmed that many aged persons who do not have dementia and who are aging "normally" show AD pathology (Bennett et al., 2006; Price et al., 2009). Neurofibrillary tangles do not correlate well with neural death as dying neurons often do not contain tangles (Alzheimer, 1907; Andorfer et al., 2005; Gómez-Isla et al., 1997; Spires et al., 2006). Additionally, a novel mutation of both APP and Aβ has been found which causes dementia and synaptic deficits without the accumulation of neuritic plaques (Blennow et al., 2006; Tomiyama et al., 2010). Transgenic mice with increased Aβ levels often experience cognitive deficits before plaques form, if they form at all (Ferri et al., 2005; Lesné et al., 2006; Westerman et al., 2002). Multiple studies have demonstrated that soluble tau, rather than insoluble tau found in neurofibrillary tangles, is responsible for neurotoxic effects (Ferri et al., 2005; Oddo et al., 2006; Santacruz et al., 2005). These results downplay the importance of plaques and tangles in AD progression focusing the attention of researchers towards earlier time points in the disease and soluble species of Aβ and tau.

C. Of Mice and Men: Pushing AD researchers towards the synapse

The creation of transgenic mouse models of AD has both advanced the study of the disease and lead to new questions. The genetic alterations in mouse models have proved quite capable at increasing Aβ levels and undergo neuritic plaque formation, but the degree of widespread neuronal loss reported has been marginal even at time points

when behavioral deficits are detected (Alzheimer, 1907; Blennow et al., 2006; Bondolfi et al., 2002; Games et al., 1995; Hsiao et al., 1996; Rupp et al., 2011; Zahs and Ashe, 2010). The low amount of neuronal loss in transgenic mice expressing high levels of Aβ led groups to hypothesize that behavioral deficits are initially the result of synaptic dysfunction rather than cell death. A number of groups have investigated synaptic changes in the hippocampus. Widespread synapse loss has long been identified in the brain tissue of dementia patients, often via the imaging of the presynaptic terminal marker synaptophysin (Arendt, 2009; Blennow et al., 2006; Davies et al., 1987; Gonatas et al., 1967; Heinonen et al., 1995; Masliah et al., 1989). In fact, these synaptic changes have been found to have a stronger correlation with cognitive decline than plaque or tangle load in humans with dementia (Davies et al., 1987; DeKosky and Scheff, 1990; Masliah et al., 1989; Masters et al., 1985; Selkoe, 2002; Sze et al., 1997; Terry et al., 1991). Loss of synapses has also been identified in multiple AD transgenic mice as early as 2 months of age (Haass et al., 1992; Hsieh et al., 2006; Lanz et al., 2003; Mucke et al., 2000; Perez-Cruz et al., 2011). As early as 3 weeks of age, baseline synaptic properties (AMPA and NMDA glutamate receptor currents) are altered in AD transgenic mice (Blennow et al., 2006; Hsia et al., 1999; Hsieh et al., 2006; Kamenetz et al., 2003; Roberson et al., 2011). Various transgenic mice lines have a deficiency in long term potentiation (LTP) of hippocampal synapses found at 8 months of age and later (Chapman et al., 1999; Grundke-Iqbal et al., 1986; Gureviciene et al., 2004; Nukina and Ihara, 1986). LTP is a long-researched cellular model of learning that results in the strengthening of synapses after a battery of activity (Malenka and Bear, 2004). Research

using AD transgenic mice shows that synapses can be affected at early time points in the disease and suggests that these changes may be related to the progression of dementia.

Synaptic deficits in Aβ mouse models often do not correlate well with plaque formation, but do correlate with elevations in soluble Aβ itself (Kovacs et al., 2008; McLean et al., 1999; Näslund et al., 2000; Oddo et al., 2003; Selkoe, 2002; Sheng et al., 2012). Moreover, genetic mutations correlated with AD in humans are associated with an increase in the levels of Aβ or an increase in the proportion of $A\beta_{1-42}$ to $A\beta_{1-40}$ (Two common truncations of the protein found in humans) (Blennow et al., 2006; Fotuhi et al., 2009; Langa et al., 2004; Schneider, 2009; Tanzi and Bertram, 2005). Recent research has shifted to understanding the effect of $A\beta_{1-42}$ on synapses and the findings have reframed the amyloid hypothesis to focus upon soluble Aβ oligomers (also referred to as Aβ-derived diffusible ligands (ADDLs) or Aβ micro-aggregates), rather than neuritic plaques (Arriagada et al., 1992; Haass and Selkoe, 2007; Krafft and Klein, 2010; Sheng et al., 2012). Soluble Aβ oligomers can be generated from multiple sources, including synthetic peptides, cell culture media from AD transgenic mice, and even human brain tissue from dementia patients. Given the variety of compositions that soluble Aβ oligomers can assume, it is not surprising that differences in the synaptic effects of the various oligomers have been reported (Larson and Lesné, 2012). One such soluble Aβ oligomer, Aβ*56 has been shown to cause cognitive deficits in mice (Cheng et al., 2007; Lesné et al., 2006). Numerous studies have shown that soluble Aβ oligomers cause the collapse of spines in hippocampal cell and brain slice cultures after 5-15 days of treatment (Hsieh et al., 2006; Lacor et al., 2007; Shankar et al., 2007; 2008; Shrestha et

al., 2006). Soluble Aβ oligomers have also been shown to impair the expression of LTP in the hippocampus at time points <1 hour (Hsieh et al., 2006; Lacor et al., 2007; Shankar et al., 2007; 2008; Shrestha et al., 2006). Electrophysiological and immunocytochemical studies have shown that treatment with soluble Aβ oligomers leads to deficits in both AMPA and NMDA glutamate receptor signaling and expression at the synapse (Chen et al., 2002; Hsieh et al., 2006; Hulette et al., 1998; Shankar et al., 2007; Snyder et al., 2005; Zhao et al., 2010). The recapitulation of synaptic changes found in AD transgenic mice by the treatment of cultured neurons with soluble Aβ oligomers validates the hypothesis that Aβ oligomers are responsible for these changes. Furthermore, the use of soluble Aβ oligomers in culture provides an excellent model to probe the cellular and molecular mechanisms by which synapses are affected – a model that we will use in Chapter 2.

D. It Takes Tau to Tangle: Evidence supporting the role of tau as the mediator of AD

Tau is the primary protein found in neurofibrillary tangles and the second pathological hallmark of AD. Due to years of research indicating that Aβ mediates AD pathogenesis clinicians seeking to develop treatments have focused on Aβ interventions (Bennett et al., 2006; Hardy and Selkoe, 2002; Price et al., 2009; Tanzi and Bertram, 2005). Unfortunately, the results of drug trials that target Aβ have not yielded substantive results (Gilman et al., 2005; Green et al., 2009). This may be due to the highly varied pathology found in patients with a clinical diagnosis of AD, but it has

broadened the range of proteins that clinicians target, this is especially true for tau (Golde et al., 2010). A growing body of literature is now implicating tau as necessary and sufficient to drive cognitive impairment in animal models and ostensibly AD in humans. Aβ may still play a role in AD pathogenesis, but as an initiator of tau-mediated dementia rather than an effector itself.

Transgenic mouse models in which tau is mutated cause synaptic and cognitive deficits that are similar to those seen in transgenic mouse model that increase soluble Aβ levels (Hoover et al., 2010; Ramsden et al., 2005; Santacruz et al., 2005). When a P301L mutant tau transgene is turned off for a period of time mice recover from the cognitive deficits found while the transgene is active, indicating the necessity of the mutant tau for deficits (Santacruz et al., 2005). The pathology found in transgenic tau mice is exacerbated by increased Aβ levels (Bolmont et al., 2007; Götz et al., 2001; Lewis et al., 2001). In the 3xTg-AD mouse expressing the APP_{Swe}, tau_{P301L}, and $PS1_{M146V}$ transgenes, Aβ pathology is detected before tau pathology suggesting the possibility of a causal relationship between the two proteins (Oddo et al., 2003; Santacruz et al., 2005). In this mouse severe cognitive deficits are not observed until tau pathology is detected (Billings et al., 2005; Oddo et al., 2004) and cognitive deficits are only mitigated when both Aβ and tau, not Aβ alone, are reduced via immunotherapy (Oddo et al., 2006). Moreover, reduction of tau in an AD mouse expressing human APP prevented cognitive deficits without affecting elevated Aβ plaque levels (Roberson et al., 2007). In dementia patients the correlation of Aβ with cognitive deficits has been found to be dependent

upon tau (Bennett et al., 2004). The combined works of these studies strongly suggest that tau is necessary for and mediates the synaptic and cognitive deficits found in AD.

Further evidence has been uncovered that shows that tau is necessary for synaptic deficits in AD transgenic mice. Deficits in axonal transport caused by the treatment of cultured neurons with soluble Aβ oligomers are dependent on the presence of tau (Vossel et al., 2010). Deficits in AMPA glutamate receptor signaling, deficits in LTP, and cognitive impairments in hAPPJ20/Fyn transgenic mice have been demonstrated to require the presence of tau (Roberson et al., 2011). The necessity of tau for soluble Aβ oligomer-induced impairment of LTP has also been shown recently using tau knockout mice (Shipton et al., 2011). Similarly, tau has been shown to be necessary for LTP deficits and cognitive impairments in tau_{RD} mutant mice (Sydow et al., 2011). These various studies implicate tau as necessary for synaptic and cognitive deficits found in AD. Moreover, these findings support a hypothetical model of AD in which soluble Aβ initiates neurodegeneration that is mediated by soluble tau.

Given that tau is a microtubule-binding protein that is typically associated with axonal processes, the necessity of tau for postsynaptic signaling deficits initially appears confounding. Tau is normally found in a gradient along the length of neurons with the highest concentrations of tau found in the axonal compartment of healthy neurons, but mutations in tau or overexpression of human tau cause the gradient to reverse and result in the misclocalization of tau to the somatodendritic compartment (Brandt et al., 2005; Gendron and Petrucelli, 2009; Götz et al., 1995; Kins et al., 2001; Papasozomenos and Binder, 1987; Spittaels et al., 1999). Recent evidence has indicated that this gradient

reversal of tau occurs in transgenic mice models of AD. A truncation of tau that prevents dendritic tau localization was found to prevent cognitive deficits in AD transgenic mice without affecting increased Aβ levels (Ittner et al., 2010). Subsequent research demonstrated that soluble Aβ oligomers cause mislocaliation of tau to dendrites and that synaptic deficits are only found in neurons demonstrating tau mislocalization (Zempel et al., 2010).

Our group has shown that tau mislocalizes to dendritic spines in tau_{P301L} mice, which mimic a mutation found in humans with frontotemporal dementia. Prevention of the mislocalization of tau in this model reverses synaptic deficits (Hoover et al., 2010). The observation that tau mislocalizes to dendrites in models of AD and dendritic spines in a model of frontotemporal dementia – along with the relationship between tau mislocalization and synaptic plasticity – bolsters the evidence that tau mediates the neurotoxic effects found early in AD. We hypothesized that tau mislocalizes to dendritic spines in AD. In Chapter 3 we will describe our research into the role of tau mislocalization in an *in vitro* model of AD.

E. The Role of Tau in Parkinson's Disease

Given research implicating tau in a wide number of dementias, we hypothesized that the mislocalization of tau to dendritic spines may represent a common pathway underlying synaptic deficits early in the disease process. Behind AD Parkinson's disease (PD) is the most common neurodegenerative malady (Alves et al., 2008; Meissner et al., 2011). PD is characterized by motor disturbances that are brought on by the loss of

striatal dopamine neurons (Lotharius and Brundin, 2002; Marsden and Fahn, 1982). Less is known about the cellular and molecular mechanisms that drive the secondary symptoms of PD which include dementia (Aarsland et al., 2008; Reichmann et al., 2009; Svenningsson et al., 2012).

Lewy bodies are the pathological hallmark of PD; their main constituent is α-synuclein (αSyn) (Goedert, 2001; Spillantini et al., 1998; 1997). For many years the physiological role of αSyn was unknown, but recent work has indicated that it is involved in presynaptic vesicle processes (Burré et al., 2010; Nemani et al., 2010). A familial strain of PD has been associated with the autosomal dominant αS point mutation A53T (Polymeropoulos et al., 1997). Transgenic mice expressing A53T αSyn display αSyn aggregation, motor deficits, neuronal loss, and impairment of neuronal function (Dev et al., 2003; Lee et al., 2002). Recent evidence indicates that the presynaptic role of αSyn in SNARE-complex assembly is not inhibited by the A53T αSyn mutation; however, the mutation increases neurotoxicity (Burré et al., 2012).

It has been suggested that tau plays a role in PD (Goris et al., 2007). Tau is found in Lewy Bodies along with αSyn (Avila et al., 2004; Ishizawa et al., 2003). Tau is hyperphosphorylated in brain samples from PD patients; *in vitro* studies indicate that exposure of tau to αSyn promotes the phosphorylation of tau (Duka et al., 2009; Haggerty et al., 2011; Kawakami et al., 2011). Tangle-like tau inclusions are found in both mouse and human subjects expressing A53T αSyn (Giasson et al., 2003; Kotzbauer et al., 2004). Given our findings implicating tau hyperphosphorylation, tau mislocalization to dendritic spines, and synaptic deficits in frontotemporal dementia

(Hoover et al., 2010), we hypothesized that neuronal expression of A53T αSyn causes synaptic deficits in hippocampal neurons by inducing the mislocalization of tau to dendritic spines. Our novel finding supporting this hypothesis will be described in Chapter 4.

Chapter 2: Differential modulation of morphine-induced structural and functional plasticity of dendritic spines

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Authorship Contributions

Participated in research design: Miller, Loh, Law, and Liao.
Conducted experiments: Miller, Zhang, Dummer, Cariveau, and Liao.
Performed data analysis: Miller, Zhang, Dummer, Cariveau, and Liao.
Wrote or contributed to the writing of the manuscript: Miller and Liao.

I. Introduction

Forms of synaptic plasticity such as LTP and LTD are widely studied as cellular models of learning and memory (Malenka and Bear, 2004). Extensive study has revealed that LTP and LTD cause two major forms of synaptic plasticity: functional plasticity of excitatory synaptic transmission and structural plasticity of dendritic spine morphology and number (Cingolani and Goda, 2008; Greger and Esteban, 2007; Tada and Sheng, 2006; Wang et al., 2007). Addiction has been proposed to be a pathological form of learning (Hyman et al., 2006) and exposure to various drugs of abuse has been found to cause both morphological plasticity of dendritic spines and functional plasticity of excitatory synaptic transmission (Bowers et al., 2010; Robinson and Kolb, 2004; Russo et al., 2010; Wolf, 2010).

Our previous work has demonstrated that chronic treatment with morphine causes collapse of dendritic spines and suppresses excitatory synaptic transmission in hippocampal neurons in a post-synaptic, μ-opioid receptor (MOR) dependent manner (Liao et al., 2007a; 2005; 2007b). Although dendritic spine plasticity and synaptic AMPA receptor plasticity have been shown to be highly correlated in a number of studies (Matsuzaki et al., 2004; McKinney et al., 1999; Okamoto et al., 2009; Richards et al., 2005), other studies have found that changes in spine morphology and synaptic AMPA receptors can be differentially mediated (Sdrulla and Linden, 2007; Wang et al., 2007). Until the present study, it has been unknown whether these two cellular processes can be differentially mediated in drug-induced plasticity of excitatory synapses.

Ca^{2+}/calumodulin-dependent protein kinase II (CaMKII) is a serine/threonine kinase that is widely-known for its critical roles in synaptic plasticity and learning and memory (Lledo et al., 1995; Okamoto et al., 2009; Silva et al., 1992). Chronic morphine treatment decreases the phosphorylation activity of CaMKII *in vivo* (Lou et al., 1999) and *in vitro* (Zheng et al., 2010). Inhibition of CaMKII in the hippocampus blocks the development of morphine tolerance and dependence (Fan et al., 1999; Lu et al., 2000). Calcineurin is a phosphatase that has recently been reported to be necessary for morphine-induced internalization of the GluR1 AMPA receptor subunit (Kam et al., 2010). Overexpression of calcineurin *in vivo* has been shown to impair morphine reinforcement (Biala et al., 2005). Despite extensive previous studies supporting the role of both signaling proteins in morphine addiction, their role in morphine-induced plasticity of dendritic spines has not yet been fully revealed.

Here we report that divergent pathways mediate morphine-induced spine loss and the loss of synaptic AMPA receptors. The inhibition of CaMKII at its auto-phosphorylation site, Thr286, blocks the effect of morphine on spine morphology, but not the effect of morphine on AMPA receptors. In contrast, the inhibition of calcineurin phosphatase activity blocks both morphine-induced spine loss and the loss of synaptic AMPA receptors. These results together provide the novel finding that divergent pathways mediate the structural and functional plasticity of excitatory synapses by morphine, shedding new light on the cellular mechanisms that may underlie opiate-induced behavioral changes.

II. Results

A. Inhibition of Both CaMKII and Calcineurin Prevents Morphine-Induced Spine Collapse

Our previous studies have demonstrated that chronic postsynaptic activation of MORs leads to two forms of plasticity of dendritic spines: loss of pre-existing spines and loss of synaptic AMPA receptors (Kam et al., 2010; Liao et al., 2007a; 2005; 2007b). Here, we used our time-lapse live imaging system to clarify the signaling pathway that mediates morphine-induced spine loss (Figure 1). MOR activity post-synaptically modulates the structural plasticity of dendritic spines (Liao et al., 2007a; 2005). CaMKII and calcineurin are known to play an important role in structural plasticity of dendritic spines during LTP and LTD (Matsuzaki et al., 2004; Silva et al., 1992). However, their roles in drug-induced morphological changes in spines remain unknown. To test their roles in MOR-mediated spine plasticity, we blocked CaMKII kinase activity with KN-62 (Tokumitsu et al., 1990) and calcineurin phosphatase activity with FK506 (Lieberman and Mody, 1994). Cultured rat hippocampal neurons at 21 DIV were treated by adding vehicle, morphine (10μM), morphine plus KN-62 (10μM), morphine plus FK506 (1μM), KN-62 alone or FK506 alone to the culture media for the length of the experiment (Figure 1A). In past studies we have found that vehicle treatment alone has no significant effect on dendritic spine density (Liao et al., 2005). Dendritic protrusions with a head 50% wider than its neck are defined as dendritic spines. Morphine treatment significantly decreased both spine and total protrusion density (number/100 μm length of dendrites) 1-

3 days after treatment (Figure 1B and 1C; n=8-10 neurons/group, $p < 0.01$; repeated measures one way ANOVA). Treatment with either KN-62 or FK506 alone had no significant effects on the density of dendritic spines (Figure 1B) or protrusions (Figure 1C). The presence of KN-62 or FK506 prevented the changes in both spine and protrusion density caused by morphine (Figure 1B and 1C; Before vs. Day 1 Morphine and No Drug vs. Day 3 Morphine, $p<0.001$, n=10). We believe that the application of KN-62 prevents the decrease in CaMKII activity that is caused by morphine treatment (Zheng et al., 2010) and that FK506 prevents increases in calcineurin activity caused by morphine treatment (Kam et al., 2010). These results indicate that the activation of both CaMKII and calcineurin is necessary for morphine-induced changes in spine density.

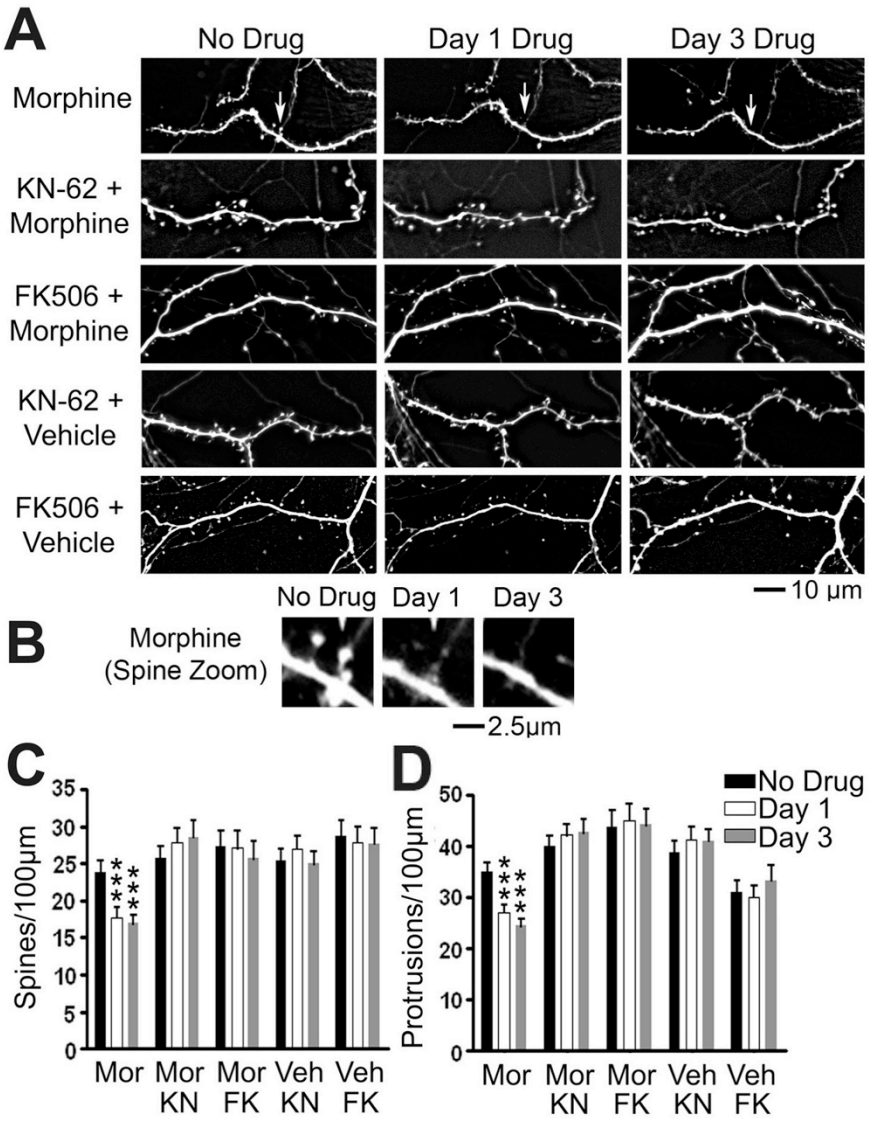

Figure 1. Inhibition of Both CaMKII and calcineurin prevents morphine-induced spine collapse

A. Representative live images of GFP-labeled cultured hippocampal neurons first taken at 21 DIV and then imaged again 1 and 3 days after drug treatment. Scale Bar = 10 µm.

B. Zoomed image of a collapsing spine from the morphine treated lane of panel A. Scale Bar = 2.5 µm.

C. Quantification of spine density per 100 µm of dendritic length in neurons described in A. Morphine treatment causes a significant decrease in spine density that is blocked by co-treatment with KN-62 or FK506.

D. Quantification of protrusion density of neurons described in A. Morphine treatment causes a significant decrease in protrusion density that is blocked by co-treatment with KN-62 or FK506.

Repeated Measures Two-way ANOVA, Bonferroni post-test, ***$p<0.001$

B. Expression of Dominant-Negative Mutant CaMKII Prevents Morphine-Induced Spine Collapse

To further explore the necessity of CaMKII for MOR-induced structural plasticity, we enlisted the use of a dominant negative CaMKII (dnCaMKII) construct with a point mutation at autophosphorylation site Thr286 (see Giese et al, 1998; a generous gift from Dr. Richard Huganir, John Hopkins University). Mice expressing CaMKII with this mutation show disruptions in spatial learning and hippcampal CA1 LTP (Giese et al., 1998). At 7 DIV, we transfected cultured hippocampal neurons with a plasmid encoding GFP alone, two plasmids encoding GFP-tagged wild-type CaMKII (wtCaMKII) and DsRed, or two plasmids encoding GFP-tagged dnCaMKII and DsRed. Whereas the GFP-tagged CaMKII constructs are appropriate for examining the cellular distribution of CaMKII, DsRed is more appropriate for visualizing the morphology of dendrites. Neurons were then treated with vehicle, morphine or naloxone (10μM) at 21 DIV. Naloxone is a non-specific MOR antagonist that we have shown causes an increase in spine density (Liao et al., 2005). We imaged the neurons before drug exposure, and then 1 and 3 days after drug exposure. There was no significant effect of morphine on the density of dendritic spines in neurons that had been transfected with dnCaMKII (Figure 2A-C). In contrast, morphine caused a significant decrease in both spine and total protrusion density in neurons expressing GFP or wtCaMKII after 1 and 3 day morphine treatment (Figure 2A-C). Naloxone significantly increased the density of dendritic spines and protrusions in these neurons at 1 and 3 DIV (Figure 3A-C). Naloxone did not cause a significant change in spine or protrusion density in neurons

transfected with dnCaMKII (Figure 3B and 3C; repeated measures two-way ANOVA, Interaction $p<0.01$; Bonferroni post-test used for differences between individual groups; n=8-10). These results extend the hypothesis that CaMKII kinase activity is required for opioid-induced structural plasticity.

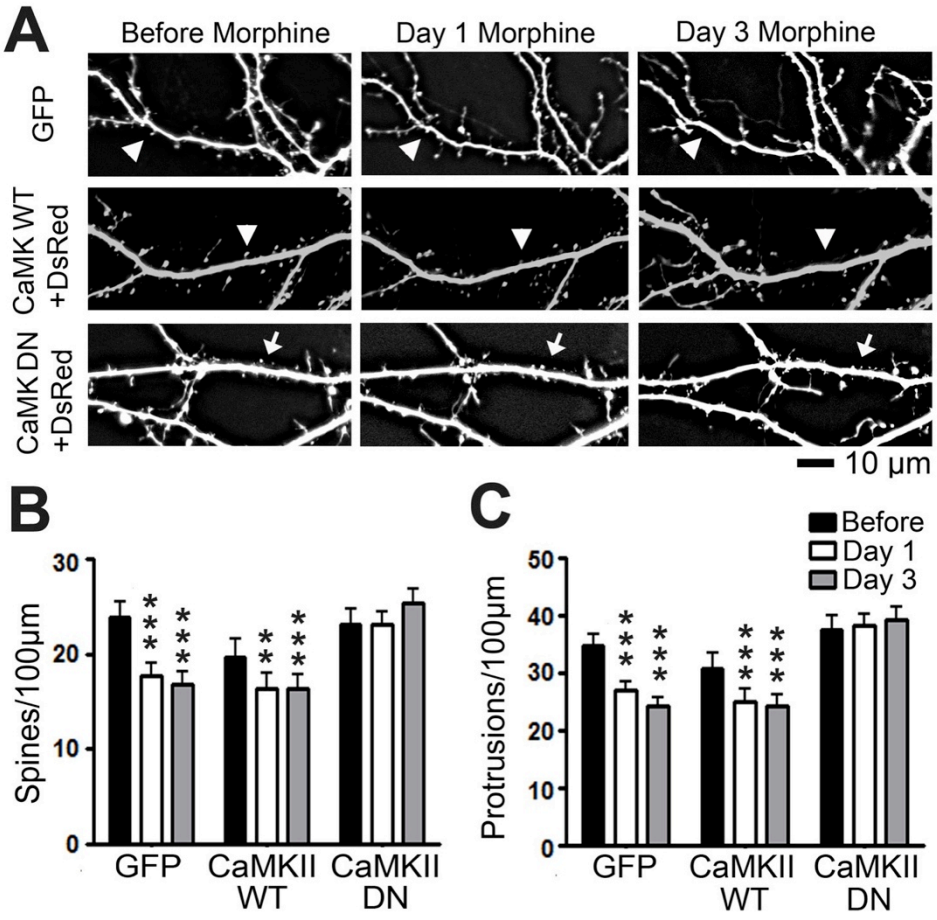

Figure 2. Expression of dominant-negative mutant CaMKII prevents morphine-induced spine collapse

A. Representative live images of cultured hippocampal neurons first taken at 21 DIV and then imaged again 1 and 3 days after drug treatment. Neurons were transfected with plasmids encoding either GFP alone, DsRed and GFP-tagged CaMKII WT, or DsRed and CaMKII DN tagged with GFP at 7 DIV. Row 1 shows GFP, Row 2 and 3 show DsRed. Arrows denote spine collapse. Scale Bar = 10 µm.

B. Quantification of spine density in A. Morphine treatment causes a significant decrease in spine density. The effect persists when neurons are transfected with CaMKII WT but is blocked when neurons are transfected with CaMKII DN.
C. Quantification of protrusion density in A. Morphine treatment causes a significant decrease in protrusion density. The effect persists when neurons are transfected with CaMKII WT but is blocked when neurons are transfected with CaMKII DN.
Repeated Measures Two-way ANOVA, Bonferroni post-test, **p<0.01, ***p<0.001

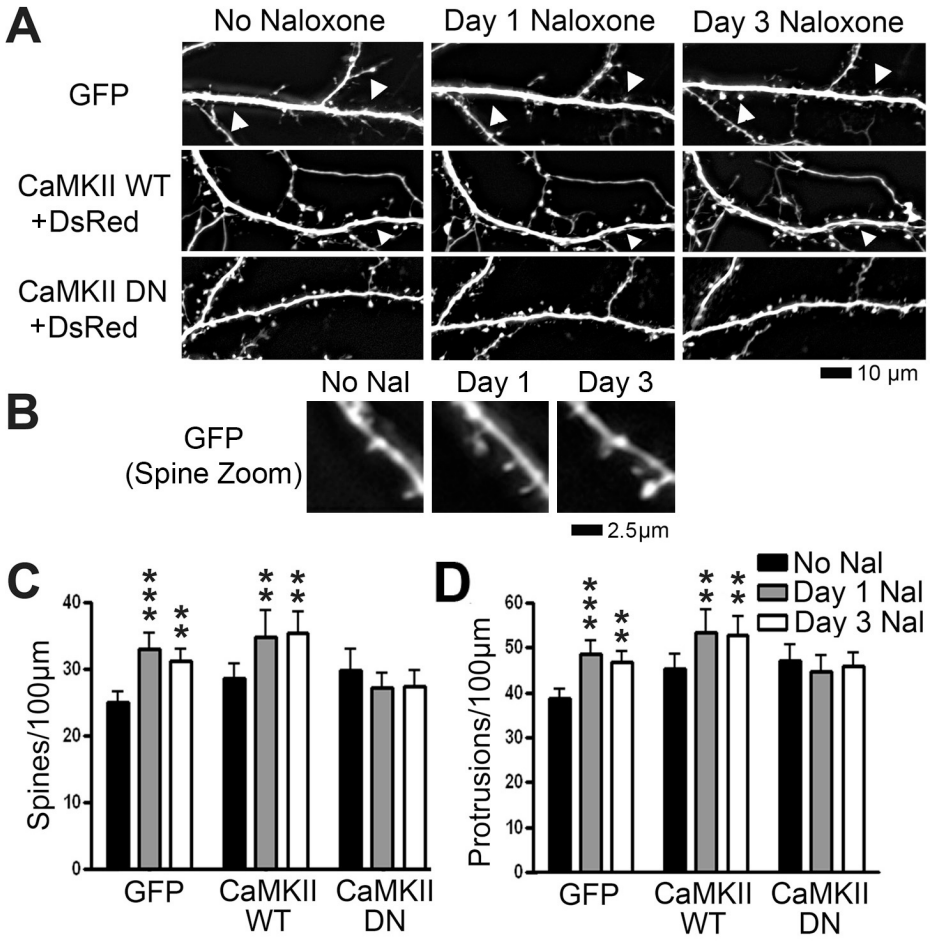

Figure 3. Expression of dominant-negative mutant CaMKII prevents naloxone-induced increases in spine density
A. Representative live images of cultured hippocampal neurons first taken at 21 DIV and then imaged again 1 and 3 days after drug treatment. Neurons were transfected with plasmids encoding either GFP alone, DsRed and GFP-tagged CaMKII WT, or DsRed and CaMKII DN tagged with GFP at 7 DIV. Row 1 shows GFP, Row 2 and 3 show DsRed. Arrowheads indicate growth of spines. Scale Bar = 10 µm.
B. Zoomed image of a new spine from the GFP-transfected, naloxone treated lane of panel A. Scale Bar = 2.5 µm.

C. Quantification of spine density in A. Naloxone treatment causes a significant increase in spine density. The effect persists when neurons are transfected with CaMKII WT but is blocked when neurons are transfected with CaMKII DN.
D. Quantification of protrusion density in A. Naloxone treatment causes a significant increase in protrusion density. The effect persists when neurons are transfected with CaMKII WT but is blocked when neurons are transfected with CaMKII DN.
Repeated Measures Two-way ANOVA, Bonferroni post-test, **p<0.01 ***p<0.001

C. Morphine Causes CaMKII Translocalization from Dendritic Spines and Dephosphorylation of CaMKII

Translocation of CaMKII to or from dendritic spines has been reported to play roles in activity-dependent structural and functional plasticity of excitatory synapses (Matsuzaki et al., 2004; Okamoto et al., 2004; Shen and Meyer, 1999; Shen et al., 2000). Therefore, it is possible that activation of MOR might also suppress CaMKII activity by suppressing the clustering of this kinase in dendritic spines. To test this possibility, we first used immunocytochemistry to determine whether MOR and CaMKII are colocalized in dendritic spines. Low-density cultured hippocampal neurons at 21 DIV were co-stained with antibodies against MOR and CaMKII using a protocol as previously described (Hoover et al., 2010; Figure 4A). We found that both proteins were highly expressed in dendrites and are largely co-localized in dendritic spines (>90%). Given that postsynaptic signaling of CaMKII can be highly localized to the dendritic spine compartment (Okamoto et al., 2009), this finding supports the hypothesis that MOR modulates structural plasticity through effects on postsynaptic CaMKII signaling in the dendritic spine.

To further test this possibility, low-density cultures of hippocampal neurons at 21 DIV were treated with MOR agonists and antagonists and co-stained with antibodies

against PSD-95 (a post-synaptic marker) and CaMKII after fixation and permeabilization (Figure 4B). Neurons were treated with vehicle, morphine, naloxone, morphine + naloxone, CTOP (10μM), or morphine + CTOP for 3 days. Morphine caused a significant decrease in clustering of CaMKII in dendritic spines, as measured by the ratio of CaMKII-expressing spines to PSD-95-expressing spines (Figure 4C; one-way ANOVA, $p<0.0001$; Bonferroni post-test, $p<0.001$; n= 9 neurons/group). The morphine-induced translocation of CaMKII from dendritic spines was blocked by either naloxone or CTOP, a MOR-selective antagonist, supporting the proposed mechanism that MOR activation by morphine suppresses synaptic CaMKII activity by either promoting the translocation of CaMKII from the postsynaptic compartment or inhibiting synaptic recruitment of the kinase.

The phosphorylation of the CaMKII auto-phosphorylation site, Thr286, is known to activate the kinase and antibodies against this phosphorylation site are commonly used to measure CaMKII activity (Giese et al., 1998). To access the effect of morphine on CaMKII phosphorylation we treated cultured hippocampal neurons with vehicle, morphine, or morphine + naloxone for 1 day and then made lysates of the cells. Our collaborators in Dr. Ping Yee Law's lab ran the lysates on a western blot and then probed for phospho-CaMKII (Thr286) and total CaMKII. We found that morphine significantly decreases phosphorylation of CaMKII and that this decrease is blocked by naloxone co-treatment with morphine (Figure 5, one-way ANOVA, $p<0.05$; Bonferroni post-test, $p<0.01$; n=4 cultures per group). These data add to previously reported data showing that morphine causes biphasic changes in CaMKII activity: acute morphine treatment (45

minutes) causes an increase in CaMKII phosphorylation (Lou et al., 1999), while chronic morphine treatment for 3 days causes a decrease in CaMKII phosphorylation (Zheng et al., 2010). Our results extend these previous findings showing that the decreasing phase of the biphasic effect of morphine on CaMKII activity occurs as early as 1 day into morphine treatment and can be blocked by the MOR antagonist naloxone. These results support our proposed model in which chronic morphine causes spine loss via a CaMKII dependent signaling pathway.

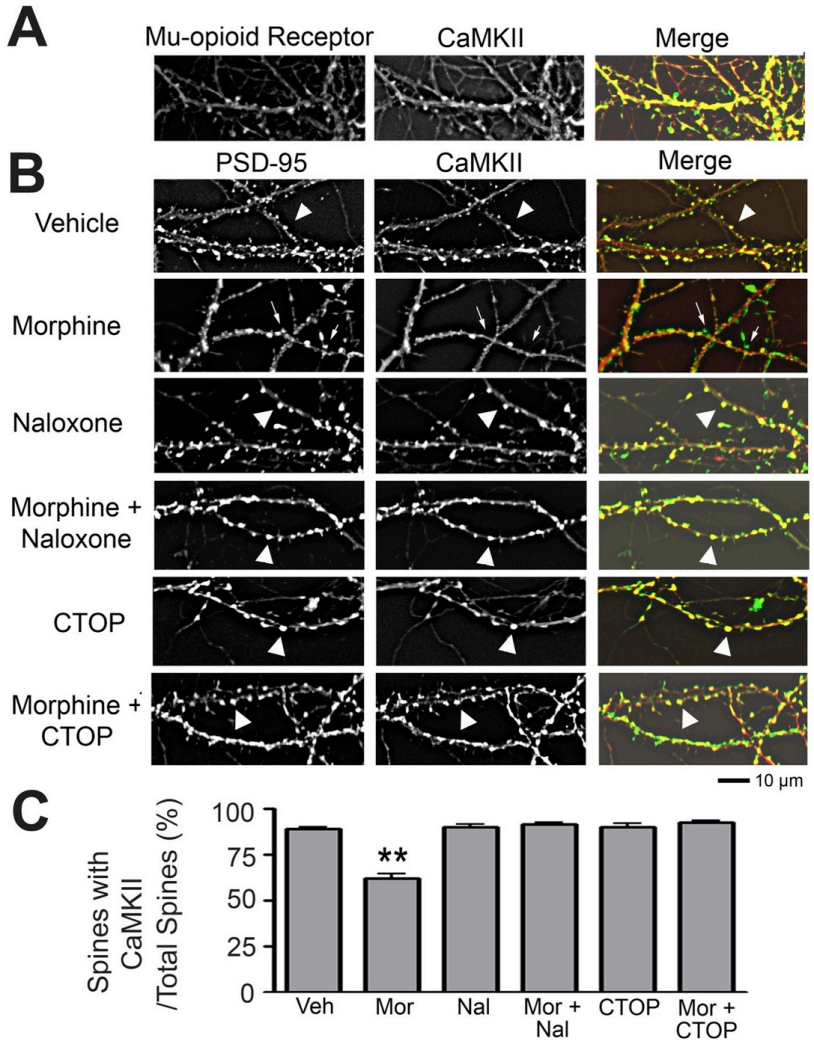

Figure 4. Morphine causes CaMKII translocalization from dendritic spines

A. Neurons stained with anti-MOR (green in the overlay) and anti-CaMKII (red in the overlay) antibodies. MOR and CaMKII colocalize in >90% of dendritic spines.
B. Representative images of neurons stained with anti-PSD-95 (green in overlay) and anti-CaMKII (red in overlay) antibodies. Neurons were treated with drug at 21 DIV and then fixed at 24 DIV. Arrowheads indicate colocalization of PSD-95 and CaMKII; arrows indicate dendritic spines where CaMKII is not found. Scale Bar = 10 μm.
C. Quantification of proportion of dendritic spines (as indicated by PSD-95) that contain CaMKII. Morphine causes the translocation of CaMKII from dendritic spines. This effect is blocked by MOR antagonists.
One-way ANOVA, Bonferroni post-test, **p<0.01

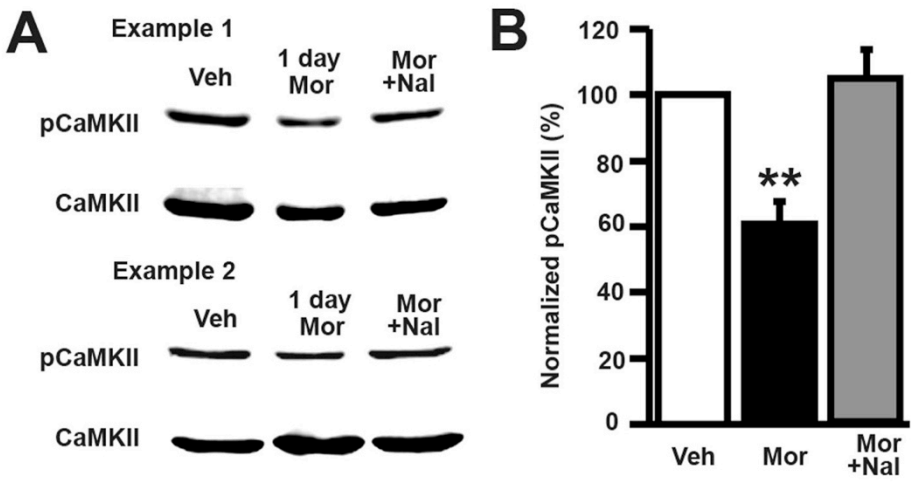

Figure 5. Morphine exposure *in vitro* causes a decrease in phosphorylated CaMKII after 1 day of treatment
A. In the two representative samples, the top rows are western blots of total cell lysates probed with a phospho-CaMKII antibody (Thr286); the bottom rows are the same lysates that have been probed with a total CaMKII antibody.
B. Quantification of the blots shown in A. Relative optical density of phosphorylated CaMKII over total CaMKII was normalized by non-treated control. Morphine decreases phospho-CaMKII and this effect is blocked by naloxone.
One-way ANOVA, Bonferroni post-test, **p<0.01

D. Rac1 is Necessary for Morphine-Induced Spine Loss

CaMKII has been shown to modulate Rac1 activity through the guanine-nucleotide exchange factor Kalirin-7 (Penzes and Jones, 2008; Xie et al., 2007). Rac1 is a GTPase that has been demonstrated to regulate the structure and function of excitatory synapses (Tashiro et al., 2000; Wiens et al., 2005). Therefore, we hypothesized that

chronic activation of MORs cause loss of pre-existing spines by inhibiting the CaMKII-Rac1 signaling pathway.

To test this possibility, we used a Rac1 activation kit (Upstate Inc.) to detect MOR-mediated changes in Rac1 activity (Li et al., 2002). High-density cultured rat hippocampal neurons at 21 DIV were treated with no drug, morphine, morphine plus CTOP or CTOP alone. We provided lysates and the Rac1 activation kit to our collaborators in Dr. Ping Yee Law's lab. To determine the amount of active Rac1 proteins, cell lysates were immunoprecipitated (IP) with PAK1, a downstream effector of Rac1, and detected using an anti-Rac1 antibody in western blots (Figure 6A). The same antibody was used to detect total Rac1 proteins in inputs of total cell lysates in the control lanes (Figure 6A). Compared with the untreated control group, morphine significantly decreased the amount of active Rac1 proteins whereas CTOP blocked the effect of morphine (Figure 5B; one-way ANOVA, Bonferroni post-test; n=4), supporting our hypothesis that CaMKII-Rac1 inhibition mediates the effects of chronic activation of MORs on dendritic spines.

To further test this hypothesis, we utilized our time-lapse live imaging system to determine how up- or down-regulation of Rac1 activity would affect the effects of morphine on dendritic spines (Figure 6C). Neurons expressing GFP, GFP-tagged constitutively active Rac1 + DsRed, and GFP-tagged dominant negative Rac1 + DsRed were imaged before, 1 day after and 3 days after morphine treatment (Figure 6). The expression of dominant negative form (Rac-) caused a significant decrease in spine density as compared to control, morphine caused no additional collapse of dendritic

spines. Transfection of the constitutively active form of Rac1 (Rac+) alone caused a significant increase in spine density, clamping spine density at a high level and preventing morphine-induced spine loss (Figure 6C-6E; repeated measures two-way ANOVA, Main effect of transfection, $p<0.0001$, Interaction $p<0.0001$; Bonferroni post-test used for differences between individual groups; n=8-10). These results demonstrate that MOR-mediated structural plasticity of dendritic spines requires changes in Rac1 activity, shedding light on the downstream mechanisms in morphine-induced spine loss.

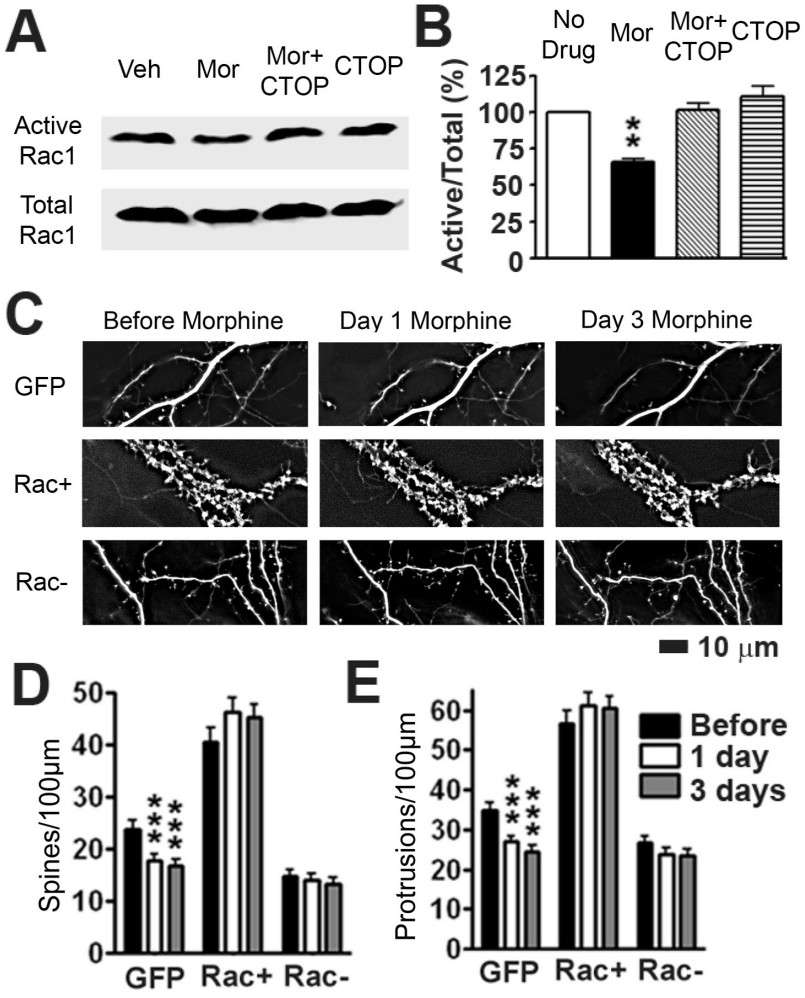

Figure 6. Rac1 is necessary for morphine-induced spine loss

A. The top row shows active Rac1 (see method). The bottom row shows input Rac1 proteins in total cell lysates.
B. Quantification of the blots shown in A. Relative optical density of active Rac1 over total Rac1 was normalized by non-treated control. Morphine decreases Rac1 activity, which is blocked by CTOP.
One-way ANOVA, Bonferroni post-test, **p<0.01
C. Representative live images of cultured hippocampal neurons first taken at 21 DIV and then imaged again 1 and 3 days after drug treatment. Neurons were transfected with either GFP, DsRed and GFP-tagged Rac+, or DsRed and GFP-tagged Rac- at 7 DIV. GFP images are displayed. For Rac+ and Rac- groups, DsRed images were used for quantification. Arrows indicate collapse of spines. Scale Bar = 10 μm.
D. Quantification of spine density in C. Morphine treatment causes a significant decrease in spine density that is blocked when neurons are transfected with Rac1- or Rac+.
E. Quantification of protrusion density in C. Morphine treatment causes a significant decrease in protrusion density that is blocked when neurons are transfected with Rac1- or Rac+.
Repeated Measures Two-way ANOVA, Bonferroni post-test, ***p<0.001

E. Calcineurin Inhibition, but Not CaMKII Inhibition, Blocks Morphine-Induced Decrease in the Amplitude of AMPAR-Mediated mEPSC Responses

As shown in Figure 1, CaMKII and calcineurin play similar roles in MOR-mediated structural plasticity of dendritic spines. Surprisingly, our electrophysiological studies revealed that these two signaling proteins play different roles in functional plasticity of dendritic spines (Figure 7). To elucidate the roles of calcineurin and CaMKII in morphine-induced functional plasticity, we used a whole-cell voltage-clamp technique to measure miniature excitatory post-synaptic currents (mEPSC) in mature cultured hippocampal neurons (21-25 DIV; Figure 7). Neurons at 19-21 DIV were treated with no drug, morphine, KN-62, KN-62 plus morphine, FK506 or FK506 plus morphine for at least 3 days in a manner similar to the morphological experiments summarized in Figure 1. Treatment with KN-62 or FK506 alone had no significant effect on mEPSC amplitude or frequency (Figure 7D and 7E). Consistent with our previous studies (Liao et al., 2007a; 2005), morphine significantly decreased the amplitude and

frequency of mEPSCs in comparison with neurons treated with vehicle (Figure 7D and 7E). The mEPSC amplitude is believed to be determined by the strength of postsynaptic response caused by the release of only one synaptic vesicle (del Castillo and Katz, 1954). Interestingly, the effect of morphine on mEPSC amplitude persisted in the presence of KN-62 (Figure 7B; Kolmogorov-Smirnoff test, $p<0.0001$; n=10) but was blocked by FK506 (Figure 7C), indicating that CaMKII and calcineurin might play different roles in modulating the trafficking of AMPA receptors into or out from dendritic spines. Consistent with analyses in Figure 7B and 7C, average mEPSC amplitude was significantly decreased by morphine treatment and this effect was blocked by FK506 but not KN-62 (Figure 7D; one-way ANOVA, n=10 in each group, compared with untreated control). In contrast, the presence of either KN-62 or FK506 blocks the effect of morphine on the frequency of mEPSCs (Figure 7E; One-way ANOVA, n=10 in each group, compared with untreated control). A decrease in the frequency of mEPSCs can result from either a decrease in releasing probability or number of synapses (subsequently decreasing number of releasing sites)(del Castillo and Katz, 1954; Liao et al., 2007a). Therefore, combined with our morphological analyses (Figure 1 and 2), our electrophysiological data indicate that both CaMKII inhibition and calcineurin activation reduce the number of functional synapses by causing collapse of dendritic spines.

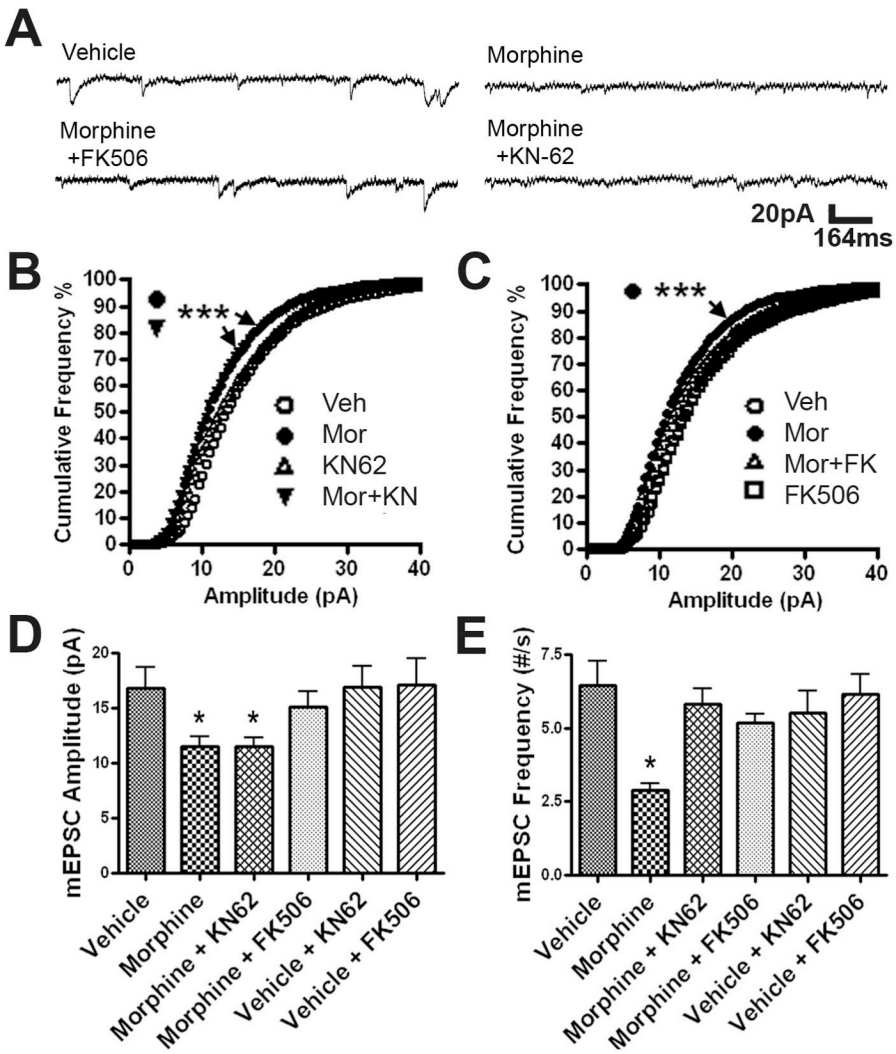

Figure 7. Calcineurin inhibition, but not CaMKII inhibition, blocks morphine-induced decrease in the amplitude of AMPAR-mediated mEPSC responses

A. Representative traces of mEPSC recordings in neurons with four different treatments. Scale Bar – x-axis=164 ms, y-axis=20 pA.

B. Cumulative frequency graph of mEPSC amplitude of neurons treated with vehicle, morphine, KN-62, and morphine + KN-62. Neurons treated with morphine and morphine + KN-62 have significantly more small amplitude mEPSCs than neurons treated with vehicle. (Kolmogorov-Smirnoff test between vehicle and morphine, between vehicle and morphine+KN-62, $p<0.0001$).

C. Cumulative frequency graph of mEPSC amplitude of neurons treated with vehicle, morphine, morphine + FK506, and vehicle + FK506. Neurons treated with morphine were found to be significantly different from vehicle (Kolmogorov-Smirnoff test between vehicle and morphine, $p<0.0001$).

D. Average mEPSC amplitudes of each group. The mEPSC amplitude of neurons treated with morphine or morphine + KN-62 was significantly decreased as compared to vehicle.

E. Average mEPSC frequencies of each group. The mEPSC frequency of neurons treated with morphine was significantly decreased as compared to vehicle.
One-way ANOVA, Bonferroni post-test, *p<0.05 ***p<0.001

F. Calcineurin Inhibition, but Not CaMKII Inhibition, Protects Against Morphine-Induced Loss of AMPA Receptors in Dendritic Spines

The morphine-induced reduction in mEPSC amplitude may result from loss of AMPA receptors in dendritic spines through endocytosis (Liao et al., 2005; Kam et al., 2010). To determine the roles of calcineurin and CaMKII in morphine-induced loss of AMPA receptors in dendritic spines, we examined the morphine-induced cellular redistribution of GluR1 and GluR2 subunits of AMPA receptors in the presence of either FK506 or KN-62 (Figures 8 and 9). Low-density rat hippocampal neurons were cultured at 21 DIV, treated with vehicle, morphine or naloxone for 3 days, and were stained with anti-PSD-95 and anti-GluR1 antibodies after fixation and permeabilization (Figure 8A-8F). We found that FK506, but not KN-62, blocked morphine-induced loss of GluR1 clustering (Figure 8G, One-way ANOVA, Bonferroni post-test, n=8 neurons/group). This indicates that calcineurin, but not CaMKII is necessary for morphine-induced removal of GluR1 from dendritic spines.

To determine whether calcineurin and CaMKII also play different roles in MOR-mediated changes in GluR2 clustering in dendritic spines, cultured neurons were treated with vehicle, morphine, morphine + FK506 or morphine + KN-62 for 3 days and then stained with anti-PSD-95 and anti-GluR2 antibodies (Figure 9A-9F). Similar to our GluR1 results, FK506, but not KN-62 blocked morphine-induced loss of GluR2 clustering (Figure 9G, One-way ANOVA, Bonferroni post-test, n=8 neurons/group).

This indicates that calcineurin, but not CaMKII is necessary for morphine-induced removal of GluR2 from dendritic spines. Consistent with our live imaging data from high-density cultures (Figure 1), FK506 and KN-62 block morphine-induced decrease in the number of PSD95 clusters in low-density cultures (Figure 9H, One-way ANOVA, Bonferroni post-test, n=8 neurons/group), further confirming that these two molecules play similar roles in MOR-mediated structural plasticity of dendritic spines.

Given that the calcineurin and CaMKII are Ca2+/calmodulin activated proteins, it is possible that the binding of morphine to MOR leads to a change in Ca2+ dynamics. Although it is unclear how morphine causes changes in intracellular Ca^{2+}, it is not likely due to an influx through NMDA channels (Kam et al., 2010). The presence of Ca2+-permeable AMPA glutamate receptors (GluR2-lacking) (CP-AMPA) at synapses has been shown to be highly plastic (Fortin et al., 2010; Liu and Zukin, 2007). CP-AMPA receptors could play a role in the effects of morphine on spine density. To test this, we transfected neurons with GFP and imaged before drug treatment, 1 day after and 3 days after. One group of neurons received morphine treatment, while the other received morphine + IEM-1460 (30μM), an inhibitor of CP-AMPA receptors. We found that co-treatment of IEM-1460 with morphine prevents morphine from causing a significant decrease in both spine and protrusion density (Figure 10, repeated measures two-way ANOVA, Bonferroni post-test, n=10 neurons/group). This finding indicates that the binding of morphine to MOR may lead to time-dependent changes in AMPA receptor subunit composition and changes in Ca2+ dynamics that cause morphine-induced spine loss.

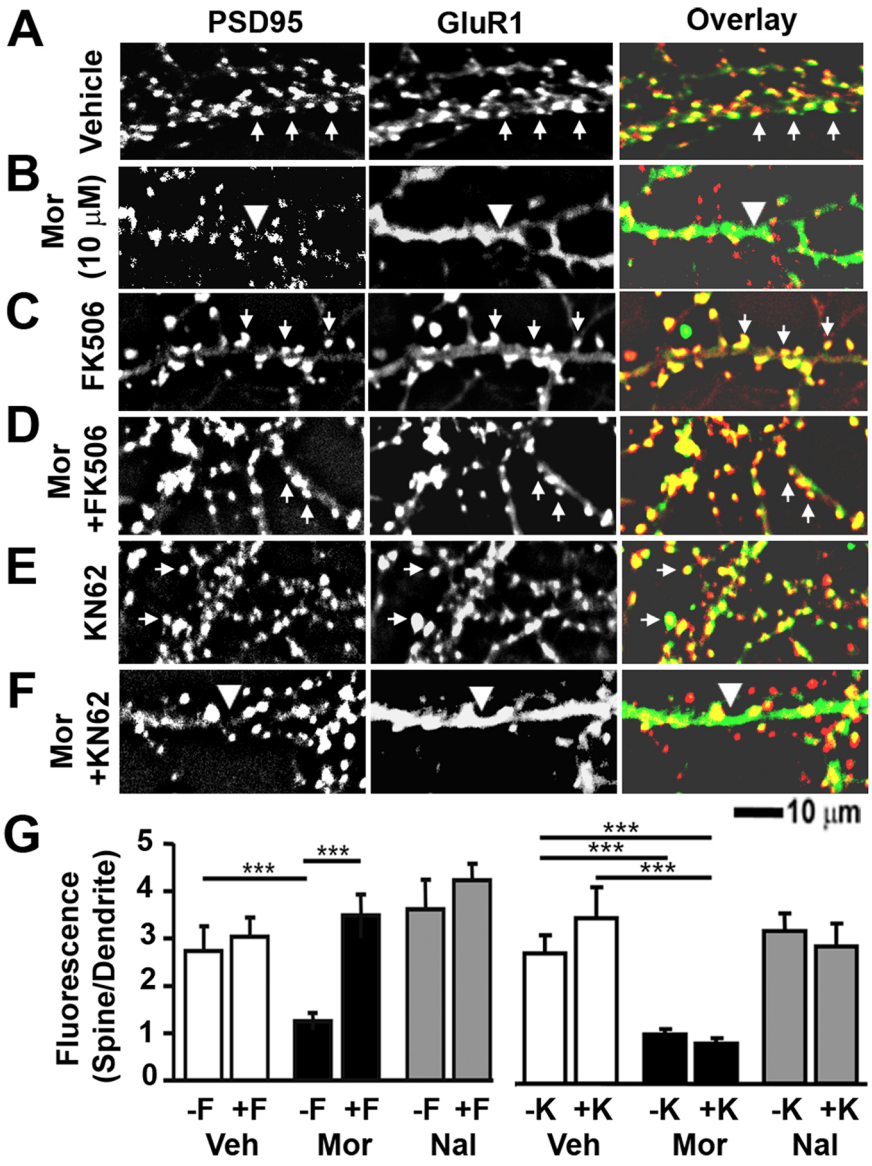

Figure 8. Calcineurin inhibition, but not CaMKII inhibition, protects against morphine-induced removal of GluR1 AMPA receptor subunits from the synapse
A-F. Representative images of neurons stained with anti-PSD-95 (red in overlay) and anti-GluR1 (green in overlay) antibodies. Arrows indicate colocalization of PSD-95 and GluR1; arrowheads indicate dendrites where GluR1 is found in the dendritic shaft. Scale Bar = 10 μm.
G. Quantification of GluR1 clustering ratio (spine/dendrite). Morphine causes the cellular redistribution of GluR1, this effect is blocked by FK506 (F), but not KN-62 (K) One-way ANOVA, Bonferroni post-test, ***p<0.001

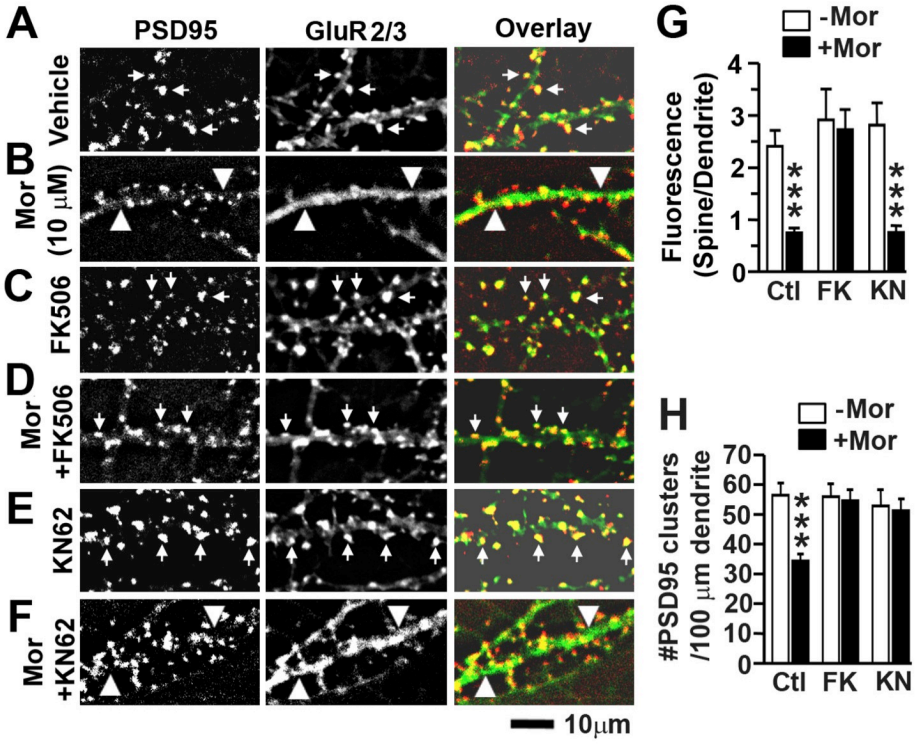

Figure 9. Calcineurin inhibition, but not CaMKII inhibition, protects against morphine-induced removal of GluR2 AMPA receptor subunits from the synapse
A-F. Representative images of neurons stained with anti-PSD-95 (red in overlay) and anti-GluR2 (green in overlay) antibodies. Arrows indicate colocalization of PSD-95 and GluR2; arrowheads indicate dendrites where GluR2 is found in the dendritic shaft. Scale Bar = 10 μm.
G. Quantification of GluR2 florescence ratio (spine/dendrite). Morphine causes the cellular redistribution of GluR2, this effect is blocked by FK506 (FK), but not KN-62 (KN) (One-way ANOVA, Bonferroni post-test).
H. Quantification of spine density by counting the number of PSD-95 clusters per 100 μm of dendritic length. Both FK506 and KN-62 prevent morphine-induced spine loss One-way ANOVA, Bonferroni post-test, ***p<0.001

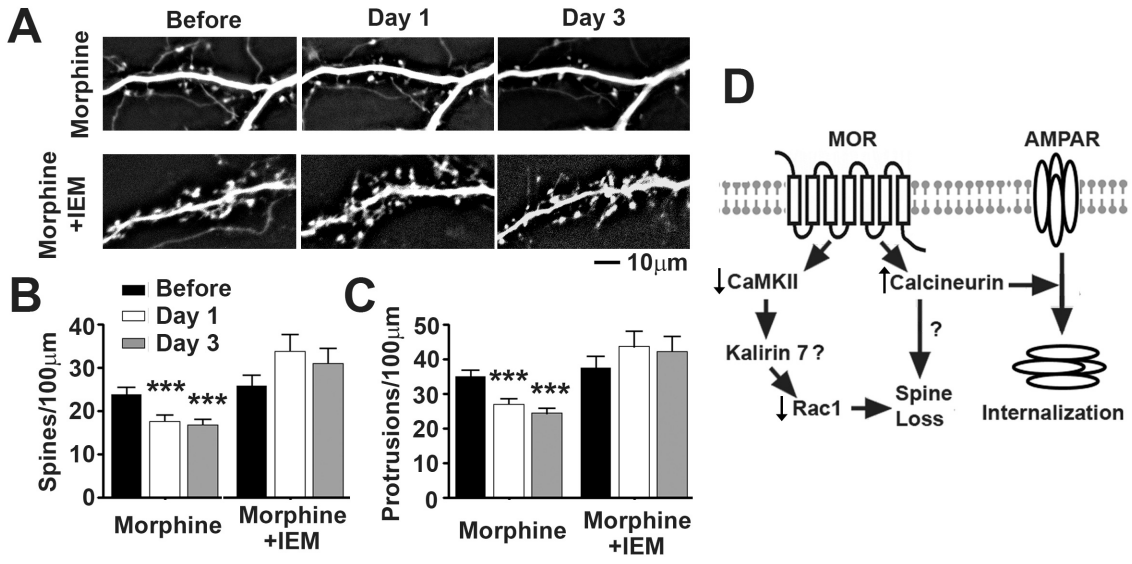

Figure 10. CP-AMPA receptor antagonist IEM-1460 prevents morphine-induced spine collapse

A. Representative live images of GFP-labeled cultured hippocampal neurons first taken at 21 DIV and then imaged again 1 and 3 days after drug treatment. Scale Bar = 10 μm.

B. Quantification of spine density per 100 μm of dendritic length in neurons described in A. Morphine treatment causes a significant decrease in spine density that is blocked by co-treatment with IEM-1460, an inhibitor of CP-AMPA receptors.

C. Quantification of protrusion density of neurons described in A. Morphine treatment causes a significant decrease in protrusion density that is blocked by co-treatment with IEM-1460.

Repeated Measures Two-way ANOVA, Bonferroni post-test, ***$p<0.001$

D. Diagram illustrating the roles of CaMKII and calcineurin in morphine-induced plasticity of dendritic spines.

III. Discussion

Persistent structural and functional changes in dendritic spines might be the basis of the abnormal learning that accompanies addiction (Bowers et al., 2010; Robinson and Kolb, 2004; Russo et al., 2010). The present study provides evidence supporting that addictive drug-induced structural and functional plasticity of excitatory synapses can be mediated through separate intracellular signaling cascades (See diagram in Figure 10D).

A. Differential Roles of CaMKII and Calcineurin in Morphine-Induced Changes in Synaptic Function

The strength of synaptic responses can be altered by changes in either quantal content (releasing probability multiplied by number of releasing sites) or quantal size (postsynaptic response per synaptic vesicle), which is often estimated by measuring the amplitude and frequency of miniature excitatory postsynaptic potential (mEPSP) or mEPSC responses (del Castillo and Katz, 1954). Surprisingly, although both calcineurin and CaMKII have been reported to play roles in AMPA receptor trafficking during activity-dependent synaptic plasticity (Dell'Acqua et al., 2006; Hayashi et al., 2000; Kam et al., 2010; Mulkey et al., 1994; Silva et al., 1992)**,** only calcineurin is necessary for morphine-induced reductions in the amplitude of AMPA receptor-mediated mEPSC responses (Figure 7). Given the assumption that amount of neurotransmitter per vesicle is constant, the decrease in mEPSC amplitude caused by morphine likely indicates a loss of postsynaptic AMPA receptors (Liao et al., 2001) and calcineurin, but not CaMKII, is necessary for that loss. This result is consistent with our previous study that chronic activation of MORs induces endocytosis of AMPA receptors by calcineurin-dependent dephosphorylation of GluR1 subunits at S845 (Kam et al., 2010). In contrast, both calcineurin and CaMKII are necessary for morphine-induced reductions in the frequency of AMPA receptor-mediated mEPSC responses (Figure 7). Given that the effect of morphine on cultured hippocampal neurons is mediated post-synaptically (Liao et al., 2007a; 2005; 2007b), morphine-induced decreases in mEPSC frequency are likely due to

dendritic spine loss, i.e. a decrease in the number of functional synapses. This finding supports our concept that calcineurin and CaMKII both participate in MOR-mediated spine loss.

B. Similar Roles of CaMKII and Calcineurin in Morphine-Induced Changes in Spine Density

Consistent with our functional analyses (see above), our time-lapse live imaging experiments revealed that both calcineurin and CaMKII are necessary for morphine-induced spine loss (Figures 1-3). CaMKII has been linked to a number of regulators of actin-binding proteins, including Rac1 and RhoA, that are involved in the structural plasticity of dendritic spines (Cingolani and Goda, 2008; Okamoto et al., 2009). One of the most widely studied regulators of spine morphogenesis is Rac1, a small GTPase. The present study provides direct evidence that the CaMKII-Rac1 signaling pathway contributes to addictive drug-induced structural plasticity of dendritic spines (Figure 6).

Ours and others' previous studies support roles of the CaMKII-Rac1 pathway in AMPA receptor trafficking during neuronal development and activity-dependent synaptic plasticity (Wiens et al., 2005; Xie et al., 2007). Surprisingly, the inhibition of CaMKII activity does not block morphine-induced changes in the amplitude of AMPA receptor-mediated mEPSC responses (Figure 7), GluR1 redistribution (Figure 8) or GluR2 redistribution (Figure 9). Taken together, these results indicate that opiate-induced changes in AMPA receptor trafficking are modulated through a signaling pathway that is different from those for neuronal development and activity-dependent synaptic plasticity.

The mechanistic link between calcineurin and CaMKII in MOR-mediated structural plasticity of dendritic spines remains to be determined. One possibility is that CaMKII acts downstream from the phosphatase calcineurin as it has been previously shown that calcineurin indirectly regulates CaMKII activity via inhibitory-1 and protein phosphatase 1 (PP1) (Colbran and Brown, 2004; Ishida et al., 2009; Lisman and Zhabotinsky, 2001). PP1 has been shown to dephosphorylate CaMKII and dissociate CaMKII from the PSD (Strack et al., 1997; Yoshimura et al., 1999), raising the possible role of calcineurin-dependent dephosphorylation of CaMKII and translocation of CaMKII from dendritic spines (Figures 4-5). Another possibility is that calcineurin-dependent AMPA receptor subunit internalization may change AMPA receptor calcium dynamics and subsequently cause spine collapse via the CaMKII-Rac1 signaling pathway (See diagram in Figure 10D).

C. Morphine-Induced Loss of GluR1 and GluR2 From Dendritic Spines Depends Upon Calcineurin, but Not CaMKII

The present study reveals that calcineurin, but not CaMKII, is necessary for morphine-induced decreases in the clustering of both the GluR1 and GluR2 subunits of the AMPA glutamate receptors in dendritic spines (Figures 8-9), further supporting the hypothesis that the two signaling molecules play different roles in morphine-induced plasticity of excitatory synapses. Recent *in vivo* research has found that 4 injections of morphine over 48 hours followed by 12 hours of withdrawal causes an increase in synaptic GluR1 AMPA receptor subunits and fEPSP magnitude (Billa et al., 2010a). Our

past and present findings indicate that after morphine has been constantly applied to the dish for 3 days *in vitro* GluR1 and GluR2 AMPA receptor subunits both cluster less in the dendritic spine and mEPSC amplitude is decreased (Liao et al., 2007a; 2005). Our lack of a withdrawal time period in our treatment protocol may be why there are differences in our groups' findings. Another explanation could be that the systematic administration of morphine leads to circuit changes that indirectly affect AMPA glutamate receptor signaling in the hippocampus.

Our previous study has shown that chronic morphine exposure increases the activity of calcineurin and that calcineurin is necessary for morphine-induced GluR1 internalization (Kam et al., 2010). As calcineurin is a Ca^{2+}-dependent protein, morphine might cause a change in intracellular Ca^{2+} to stimulate calcineurin activity. Various opioids have been shown to modulate levels of intracellular calcium and the activity of calmodulin (Nehmad et al., 1982; Smart et al., 1997). Research has indicated that mGluRs and secondary messengers PLC/PKC are involved in morphine-induced signal transduction and development of tolerance, therefore, morphine may affect intracellular Ca^{2+} levels via mGluRs (Fundytus and Coderre, 1996).

Our finding cannot determine whether the timescale of morphine-induced removal of GluR1 from dendritic spines is different from that of GluR2. GluR2-lacking AMPA receptors are Ca2+-permeable, while GluR2-containing AMPA receptors are not (Liu and Zukin, 2007). Drugs of abuse have been shown to alter AMPA receptor subunit composition and AMPA receptor Ca2+-permeability (Billa et al., 2010b; Conrad et al., 2008; Mameli et al., 2009; Wolf and Ferrario, 2010). Given the various roles of CP-

AMPA receptors in synaptic plasticity (Fortin et al., 2010; Liu and Zukin, 2007), it is possible that morphine may affect intracellular Ca^{2+} levels via modulation of AMPA receptor calcium permeability. Although data in Figure 10 support the role of CP-AMPA receptors in morphine-induced spine loss, further research must be conducted in order to determine how morphine exposure leads to increased calcineurin activity.

D. Roles of Structural and Functional Plasticity of Dendritic Spines in Hippocampal Neurons in Drug Addiction

It has been known for over two decades that unilateral microinjections of morphine into the rat hippocampus can produce a conditioned place preference (Corrigall and Linseman, 1988). Due to the classical hypothesis that the mesolimbic dopaminergic pathway is the main rewarding pathway (Bowers et al., 2010), the hippocampus has not been incorporated into the addiction neurocircuitry until very recently. Addiction is increasingly regarded as a pathological form of learning and memory (Hyman et al., 2006; Jones and Bonci, 2005). Therefore, present models of drug addiction almost always incorporate memory systems including the hippocampus, prefrontal cortex and amygdala (Koob and Volkow, 2009; Morón and Green, 2010).

Although structural and functional changes in dendritic spines during activity-dependent plasticity have been correlated in numerous studies (Matsuzaki et al., 2004; Okamoto et al., 2009), some studies have shown that the molecular mediators of functional and structural plasticity can be dissociated (Cingolani and Goda, 2008; Wang et al., 2007). The present study has extended this concept of separate but interactive

signaling pathways for mediating structural and functional plasticity of excitatory synapses to addiction research.

Persistent structural and functional changes in dendritic spines caused by drugs of abuse are recently proposed to mediate the aberrant learning associated with addiction (Hyman et al., 2006; Robinson and Kolb, 2004). Recent studies have attempted to determine the roles of synaptic plasticity in addictive drug-induced behavioral changes by either blocking cocaine-induced structural plasticity (Pulipparacharuvil et al., 2008; Russo et al., 2009), or cocaine-induced changes in glutamate receptor function (Moussawi et al., 2009; 2011). These behavioral studies often yield conflicting results (Moussawi et al., 2009; 2011; Pulipparacharuvil et al., 2008; Russo et al., 2009), which may result from the complexity of neuronal circuitry or divergent intracellular mechanisms underlying structural and functional plasticity of dendritic spines caused by drugs of abuse. The present study provides direct experimental evidence that these two forms of drug-induced plasticity can be mediated by separate, but interacting, signaling pathways. The new knowledge gained from our *in vitro* cellular studies should help us better understand the relationship between structural and functional plasticity in addiction and promote the design of more comprehensive *in vivo* experiments in the future.

E. Conclusions

The learning and memory associated with drug-induced behavioral changes requires long-lasting changes in synaptic strength, which can be either achieved through alteration in the amount of functional AMPA receptors in dendritic spines or changes in

spine morphology. The present study demonstrates that these two forms of drug-induced plasticity can be mediated through separate but interactive intracellular cascades. The new concept of separate but interactive mediators for functional and structural synaptic plasticity caused by drugs of abuse should significantly advance our understanding of the diverse cellular mechanisms underlying addictive behaviors.

Chapter 3: Soluble Aβ oligomers cause tau mislocalization to dendritic spines and calcineurin/GluR1 S845-dependent AMPA glutamate receptor internalization

Eric C. Miller, Peter J. Teravskis, Benjamin W. Dummer, Xiaohui Zhao, Richard L. Huganir, Karen H. Ashe, and Dezhi Liao. (2014). Soluble Aβ oligomers cause tau mislocalization to dendritic spines and calcineurin/GluR1 S845-dependent AMPA glutamate receptor internalization *In preparation for European Journal of Neuroscience*.

Authorship Contributions
Participated in research design: Miller, Zhao, and Liao.
Conducted experiments: Miller, Teravskis, Dummer, and Liao.
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Wrote or contributed to the writing of the manuscript: Miller, Teravskis, and Liao.

I. Introduction

Alzheimer's Disease (AD) is a dementia characterized by progressive memory loss ultimately resulting in an inability to form new memories. The pathological hallmarks of the disease are neuritic plaques, mainly composed of amyloid-β (Aβ), and neurofibrillary tangles, mainly composed of tau (Blennow et al., 2006). Synaptic deficits are highly correlated with cognitive deficits found in AD patients (Blennow et al., 2006; Davies et al., 1987; DeKosky and Scheff, 1990; Masliah et al., 1989; Selkoe, 2002; Terry et al., 1991). Synaptic deficits identified in the hippocampus of transgenic mice with increased Aβ levels include: loss of dendritic spines (Lanz et al., 2003; Mucke et al., 2000), deficits in glutamate receptor signaling (Hsia et al., 1999; Kamenetz et al., 2003; Roberson et al., 2011), and deficits in the expression of LTP (Chapman et al., 1999; Gureviciene et al., 2004). Spine loss and glutamate receptor deficits occur at early time points in transgenic mice, before cognitive decline or neuritic plaque formation is detected, suggesting that they may represent the initial stages of the disease (Bennett et al., 2006; Hulette et al., 1998).

Synaptic deficits do not correlate with plaque formation, but directly correlate with levels of soluble Aβ (McLean et al., 1999; Näslund et al., 2000; Oddo et al., 2003; Sheng et al., 2012). Furthermore, genetic mutations found in AD patients are closely associated with increased levels of $A\beta_{1\text{-}42}$ (Blennow et al., 2006). One method researchers have used to study the synaptic changes in cultures of dissociated neurons and brain slices is treatment with soluble Aβ oligomers (also referred to as Aβ-derived diffusible ligands) (Haass and Selkoe, 2007; Krafft and Klein, 2010). The method by

which soluble Aβ oligomers affect cell signaling and cause neurotoxicity is currently a point of contention (Larson and Lesné, 2012). Synaptic deficits found in AD transgenic mice have been recapitulated in cultures treated with soluble Aβ oligomers (Chen et al., 2002; Hsieh et al., 2006; Lambert et al., 1998; Shrestha et al., 2006). Soluble Aβ oligomers provide an excellent model for probing the mechanisms underlying synaptic deficits in AD.

The correlation between Aβ and cognitive deficits in AD patients is dependent upon tau levels (Bennett et al., 2004). Various studies in AD transgenic mice have demonstrated that tau is necessary and sufficient for both cognitive and synaptic deficits (Hoover et al., 2010; Oddo et al., 2006; Roberson et al., 2007; Santacruz et al., 2005; Sydow et al., 2011; Zempel et al., 2010). Although tau is most highly expressed in the axon of neurons, tau has been shown to mislocalize to the dendritic compartment in diseased states (Avila et al., 2004; Ittner and Götz, 2011; Zempel et al., 2010). Our group has extended these findings by demonstrating that tau mislocalizes to dendritic spines in tau_{P301L} mice (a model of frontotemporal dementia) and cultured hippocampal neurons when P301L tau is expressed, resulting in cognitive and synaptic deficits. Importantly, we found that phosphorylation of tau at serine/threonine residues is necessary for the mislocalization of tau and deficits in AMPA glutamate receptor (AMPAR) signaling (Hoover et al., 2010). Although we have previously demonstrated the role of tau mislocalization to dendritic spines in a model of frontotemporal dementia, it is still unknown whether tau mislocalization to dendritic spines occurs in AD.

To better understand the relationship between Aβ, tau, and synaptic deficits we investigated tau mislocalization in neurons exposed to soluble Aβ oligomers using fluorescently labeled tau. Using whole-cell voltage clamp electrophysiology we also explored the role of calcineurin (also known as PP2B) and AMPAR GluR1 residue S845 in $A\beta_{1-42}$-induced deficits in AMPAR signaling. Our findings highlight the significance of calcineurin in synaptic deficits caused by Aβ and adds further evidence to support the role of tau in AD.

II. Results

A. Tau is Mislocalized to Dendritic Spines in Neurons Cultured From APP_{Swe} Transgenic Mice

Early onset Familial Alzheimer's Disease is associated with the Swedish mutation at Aβ precursor protein (APP) 670/671 (Citron et al., 1992; Mullan et al., 1992). The APP_{Swe} mice, which express APP with Lys670Asn and Met671Leu mutations (also referred to as Tg2576), previously have been shown to have high levels of soluble Aβ oligomers that correlate with cognitive deficits (Hsiao et al., 1996; Lesné et al., 2006). To test our hypothesis we cultured hippocampal neurons from APP_{Swe} mice and their transgenic-negative (TgNg) littermates. We then transfected the neurons with WT tau tagged with GFP and DsRed at 7-10 DIV and imaged them at 21-24 DIV (Figure 11A). The transfection of these plasmids allowed us to determine the proportion of spines containing tau. We found that tau mislocalized to the dendritic spines of APP_{Swe} mice in

a significantly greater proportion than in the spines of TgNg neurons (Figure 11B; t test, $t = 4.027$, $df = 8$, $P = 0.0038$). No significant differences in spine density were found between groups (Figure 11C). These results demonstrate that tau mislocalization occurs in neurons cultured from transgenic mice that express an APP mutation found in humans with AD.

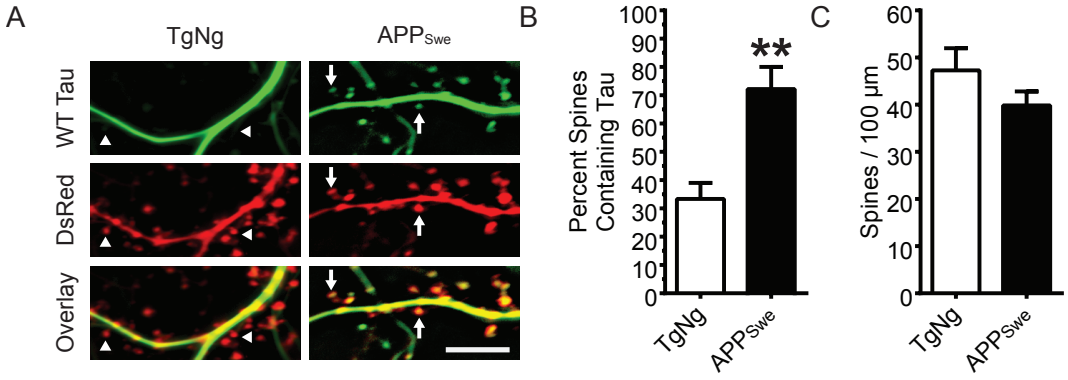

Figure 11. WT Tau is mislocalized to dendritic spines in neurons cultured from APP_{Swe} transgenic mice
A. Representative images of hippocampal neurons cultured form TgNg or APP_{Swe} transgenic mice and then transfected with WT tau (GFP) and DsRed. Images taken at 21 DIV. Arrows indicate spines that contain tau, arrowheads indicate spines lacking tau. Scale bar represent 10 µm.
B. Quantification of dendritic spines containing tau. Tau is found in a significantly greater proportion in spines of neurons from APP_{Swe} mice than TgNg mice.
C. Quantification of spine density as calculated by visual inspection of DsRed fluorescence. No significant difference was found in spine density between groups.
T test, **p < 0.01

B. Oligomerized $A\beta_{1-42}$ Causes the Mislocalization of Tau to Dendritic Spines

The above observation that mislocalization of tau to spines occurs in neurons from APP_{Swe} transgenic mice lead us to hypothesize that soluble $A\beta_{1-42}$ oligomers are responsible for the mislocalization. We prepared synthetic $A\beta_{1-42}$ in a manner that

promotes oligomerization (Hepler et al., 2006; Lambert et al., 2001; Shughrue et al., 2010). Our oligomerized synthetic $A\beta_{1-42}$ consistently contains dimers, trimers, and tetramers as measured by western blot by collaborators in Dr. Karen Ashe's laboratory (Figure 12A). The localization of tau was investigated by first transfecting cultured hippocampal neurons at 7-10 days *in vitro* (DIV) with WT tau tagged with GFP and DsRed. The GFP-tagged tau allowed us to determine the subcellular localization of tau, while the DsRed freely diffused throughout the neurons and revealed their morphology. At 21 DIV we took live images of the neurons and then treated the dishes with vehicle or oligomerized $A\beta_{1-42}$ (2 μM). The vehicle group was treated with F12 (the solvent oligomerized $A\beta_{1-42}$ is prepared in) as a control. We next imaged the same neurons one day and three days after treatment (Figure 12B). Prior to oligomerized $A\beta_{1-42}$ treatment WT tau was expressed in the dendrites and very minimally in the dendritic spines. One day and three days after treatment the proportion of spines containing tau was increased significantly (Figure 12C; repeated-measures two-way ANOVA, Interaction $F_{2,44} = 5.42$, $P = 0.0079$, Time $F_{1,22} = 7.97$, $P = 0.0099$, Treatment $F_{2,44} = 14.38$, $P < 0.0001$; Bonferroni post-test used for differences between individual groups). No significant changes were detected in the vehicle-treated group. Spine density was not significantly changed in either group (Figure 12d). These results signify that exogenous synthetic $A\beta_{1-42}$ causes tau mislocalization in cultured hippocampal neurons.

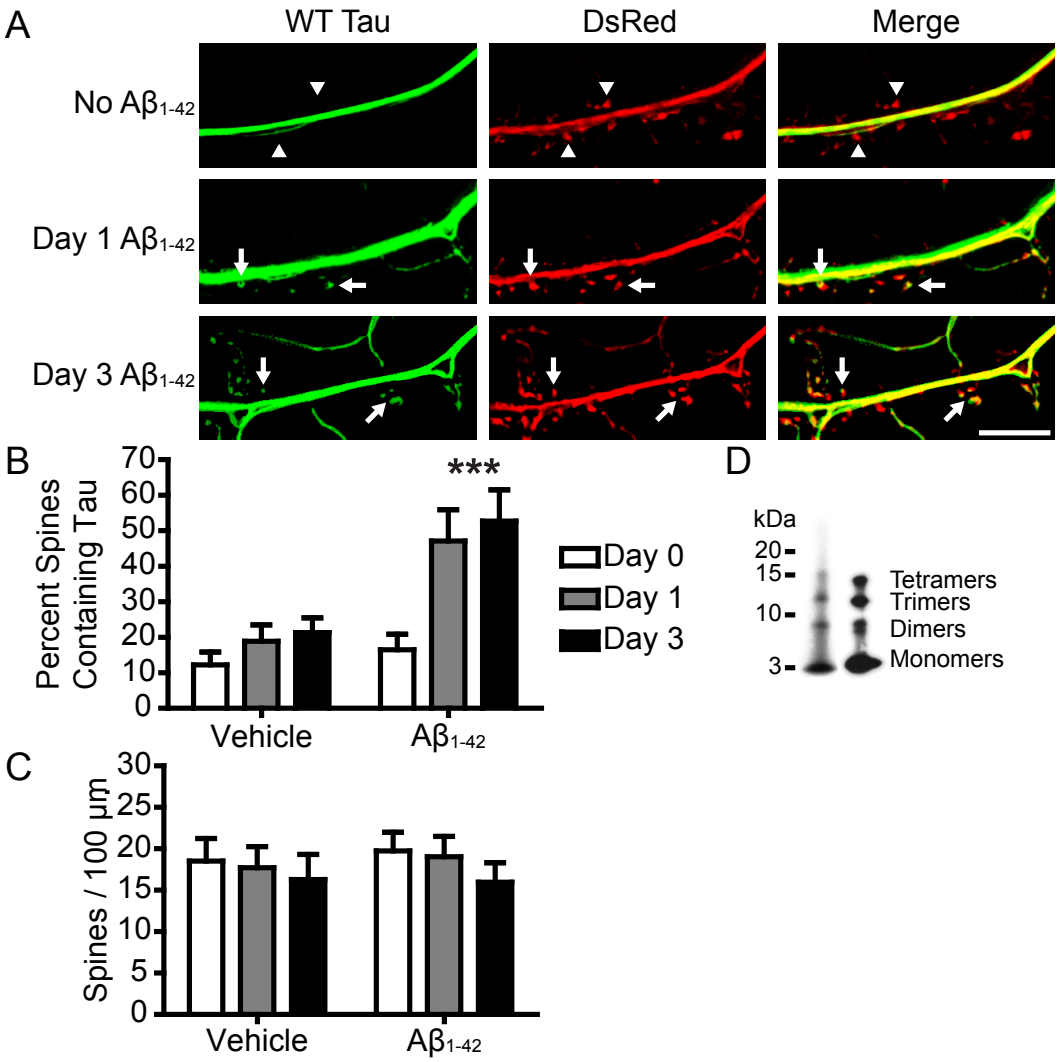

Figure 12. WT tau is mislocalized to dendritic spines in neurons treated with oligomerized $A\beta_{1-42}$

A. Representative images from cultured hippocampal neurons transfected with WT tau (GFP) and DsRed. Images taken at 21 DIV before $A\beta_{1-42}$ treatment and then 1 day and 3 days after. Arrows indicate spines that contain tau, arrowheads indicate spines lacking tau. Scale bar represent 10 µm.

B. Quantification of spines containing tau. This proportion is generated by dividing the count of spines containing tau by total spine count. Tau presence in spines is significantly increased after treatment with $A\beta_{1-42}$. Treatment with vehicle lead to no significant changes.

C. Quantification of spine density. No significant differences were found between groups.

D. Western blot showing three examples of oliomerized synthetic $A\beta_{1-42}$ that we prepared. Dimers, trimers, and tetramers are consistently found in our preparation.

Repeated-measures two-way ANOVA, Bonferroni post-test used for differences between individual groups, ***$p < 0.001$

C. Phosphorylation Activity of Tau is Necessary for Oligomerized $A\beta_{1-42}$-Induced Tau Mislocalization

We next designed an experiment to test the role of tau phosphorylation in oligomerized $A\beta_{1-42}$-induced mislocalization of tau to dendritic spines. We employed a tau construct, known as APtau, in which the 14 serine (S) and threonine (T) residues that can be phosphorylated by proline (P)-directed serine/threonine kinases (SP/TP) were mutated to alanine in order to prevent phosphorylation (Fulga et al., 2007; Hoover et al., 2010). Similar to the last experiment, we transfected cultured hippocampal neurons at 7-10 DIV with APtau tagged with GFP and DsRed. We then took live images of the neurons at 21 DIV and treated with vehicle or oligomerized $A\beta_{1-42}$. The same neurons were then imaged one day and three days after treatment (Figure 13A). In neurons transfected with APtau no change in the proportion of spines containing tau was found after oligomerized $A\beta_{1-42}$ treatment (Figure 13B, repeated measures two-way ANOVA, Bonferroni post-test used for differences between individual groups). No significant changes were detected in the vehicle group. There were no significant changes in dendritic spine density at the time points measured for vehicle or oligomerized $A\beta_{1-42}$ treated groups (Figure 13C). This experiment indicates that phosphorylation at tau SP/TP residues is necessary for oligomerized $A\beta_{1-42}$-induced tau mislocalization.

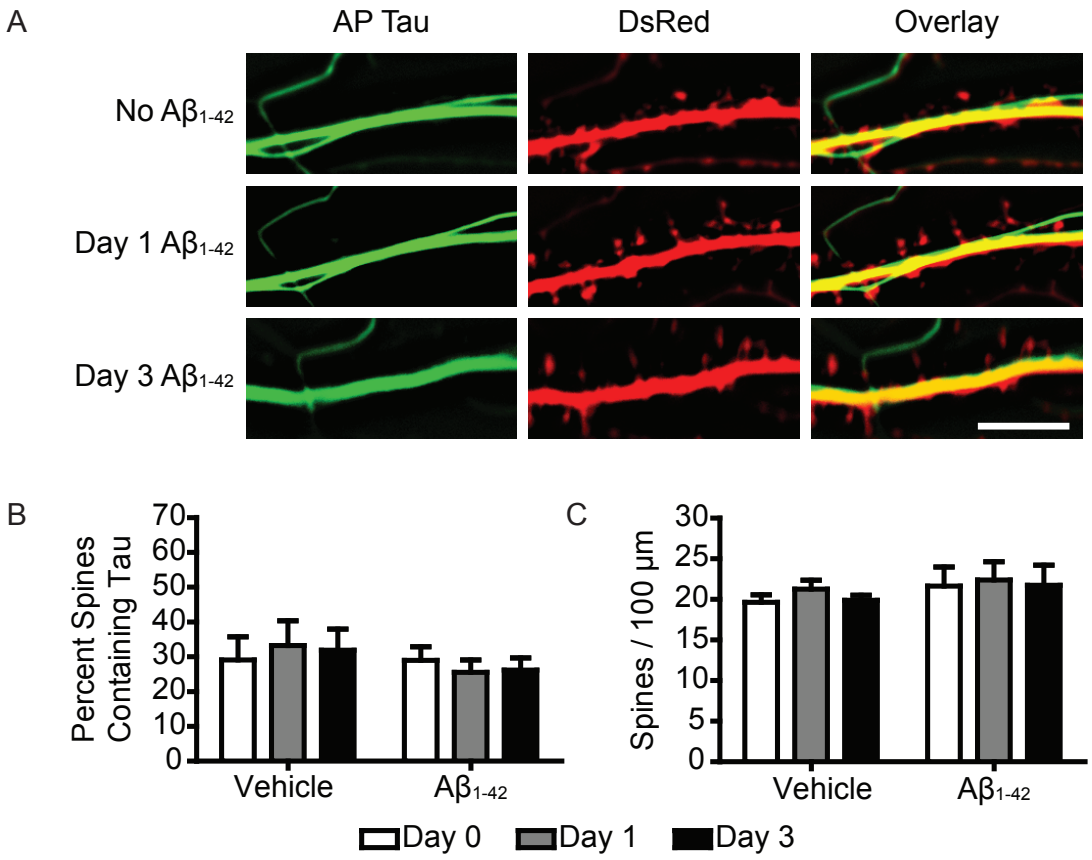

Figure 13. AP tau is not mislocalized to dendritic spines in neurons treated with $A\beta_{1-42}$

A. Representative images from cultured hippocampal neurons transfected with AP tau (GFP) and DsRed. Images taken at 21 DIV before $A\beta_{1-42}$ treatment and then 1 day and 3 days after. Scale bar represent 10 µm.

B. Quantification of spines containing tau. This proportion is generated by dividing the count of spines containing tau by total spine count. AP tau presence in spines is does not change significantly after treatment with $A\beta_{1-42}$. Treatment with vehicle lead to no significant changes as well.

C. Quantification of spine density. No significant differences were found between groups.

Repeated-measures two-way ANOVA, Bonferroni post-test used for differences between individual groups

D. Oligomerized Aβ$_{1-42}$-Induced AMPAR Signaling Deficits Require Calcineurin Activity

We next sought to investigate signaling mechanisms that mediate oligomerized Aβ$_{1-42}$-induced AMPAR signaling deficits downstream of tau. The phosphatase calcineurin has been shown to be necessary for synaptic deficits caused by soluble Aβ oligomers (Chen et al., 2002; Hsieh et al., 2006; Shankar et al., 2007; Snyder et al., 2005). Calcineurin plays an important role in AMPAR internalization and is implicated in long-term depression of hippocampal synapses (Dell'Acqua et al., 2006; Miller et al., 2012; Mulkey et al., 1994). To determine the role of calcineurin in oligomerized Aβ$_{1-42}$-induced AMPAR signaling deficits we employed the use of FK506, an inhibitor of calcineurin (Lieberman and Mody, 1994). At 19-22 DIV we treated neurons with vehicle alone, vehicle + FK506, oligomerized Aβ$_{1-42}$ alone, or oligomerized Aβ$_{1-42}$ + FK506. After three days of drug treatment we recorded AMPAR mini excitatory postsynaptic currents (mEPSC) from the cultured neurons (Figure 14A). AMPAR mEPSCs represent the activity of a spontaneously-released vesicle binding to AMPAR at a single synapse. We found that neurons treated with oligomerized Aβ$_{1-42}$ had significantly more small amplitude mEPSCs than those treated with vehicle alone; there was no significant difference between vehicle + FK506 and oligomerized Aβ$_{1-42}$ + FK506 groups (Figure 14B; Kolmogorov-Smirnov, $D = 0.5616$, $P < 0.0001$) We found that mEPSCs from neurons treated with oligomerized Aβ$_{1-42}$ had significantly lower average amplitudes than those from vehicle neurons (Figure 14C; two-way ANOVA, Interaction $F_{1,36} = 7.01$, $P = 0.012$, Aβ$_{1-42}$ $F_{1,36} = 9.33$, $P = 0.0042$, FK506 $F_{2,44} = 2.54$, $P = 0.12$; Bonferroni post-test

used for differences between individual groups). There were no significant differences in mEPSC frequency between groups (Figure 14D). These results indicate that calcineurin activity is necessary for oligomerized $A\beta_{1-42}$-induced AMPAR signaling deficits.

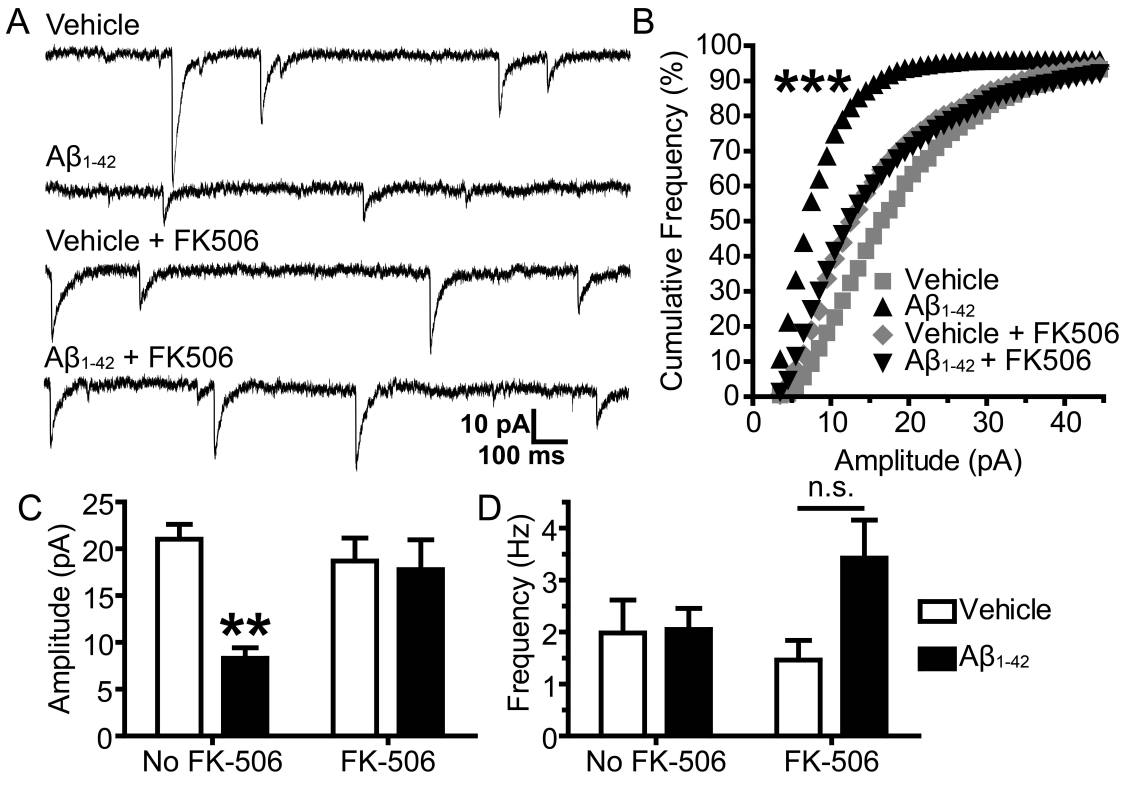

Figure 14. $A\beta_{1-42}$ treatment leads to a decrease in AMPAR mEPSC amplitude that is dependent upon calcineurin activity

A. Representative traces of AMPAR mEPSCs recorded from cultured hippocampal neurons treated with vehicle, $A\beta_{1-42}$, vehicle + FK506, or $A\beta_{1-42}$ + FK506. x-axis = 200 ms, y-axis = 20 pA.

B. Cumulative frequency distributions of mEPSC amplitudes of groups shown in (A). Neurons treated with $A\beta_{1-42}$ had significantly more small mEPSCs than neurons from the other three groups. Bin size = 1 pA.

C. Mean mEPSC amplitude of neurons shown in (A). Mean amplitude of mEPSCs from $A\beta_{1-42}$ treated neurons was significantly lower than those from the other three groups.

D. Mean mEPSC frequency of neurons shown in (A). No significant differences were found between groups.

Two-way ANOVA, Bonferroni post-test used for differences between individual groups, $^{**}p < 0.01$

E. Oligomerized $A\beta_{1-42}$-Induced Internalization of the GluR1 AMPAR Subunit Requires Activity at GluR1 S845

Calcineurin has been shown to dephosphorylate GluR1 AMPAR subunit residue S845 in cellular models that induce AMPAR internalization (Kam et al., 2010; Lee et al., 1998). We sought to determine if this GluR1 residue is necessary for calcineurin-dependent loss of AMPAR induced by oligomerized $A\beta_{1-42}$. To investigate this hypothesis we cultured hippocampal neurons from transgenic mice expressing GluR1 S845A (which renders the residue non-phosphorylatable) and their TgNg littermates. We allowed the neurons to mature in the dish and then treated them with vehicle or oligomerized $A\beta_{1-42}$ at 21 DIV. After 3 days of exposure we fixed the cells, then stained with an antibody to N-GluR1 (tagging only extracellularly expressed GluR1 subunits). We next permeablized the membranes of the fixed neurons and stained with an antibody to PSD-95 (Figure 15A&B). In neurons from TgNg mice we found that oligomerized $A\beta_{1-42}$ significantly decreased the expression of extracellular GluR1; this effect was abolished in neurons cultured from S845A transgenic mice (Figure 15C; two-way ANOVA, Interaction $F_{1,36} = 5.013$, $P = 0.031$, Genotype $F_{1,36} = 6.884$, $P = 0.013$, Genotype $F_{2,44} = 6.718$, $P = 0.014$; Tukey post-test used for differences between individual groups). These findings suggest that decreases in the amplitude of AMPAR currents found after treatment with oligomerized $A\beta_{1-42}$ are due to internalization of GluR1 AMPAR subunits. Furthermore, activity at GluR1 residue S845, likely dephosphorylation by calcineurin, is necessary for oligomerized $A\beta_{1-42}$-induced loss of GluR1 AMPAR subunits and dendritic spines.

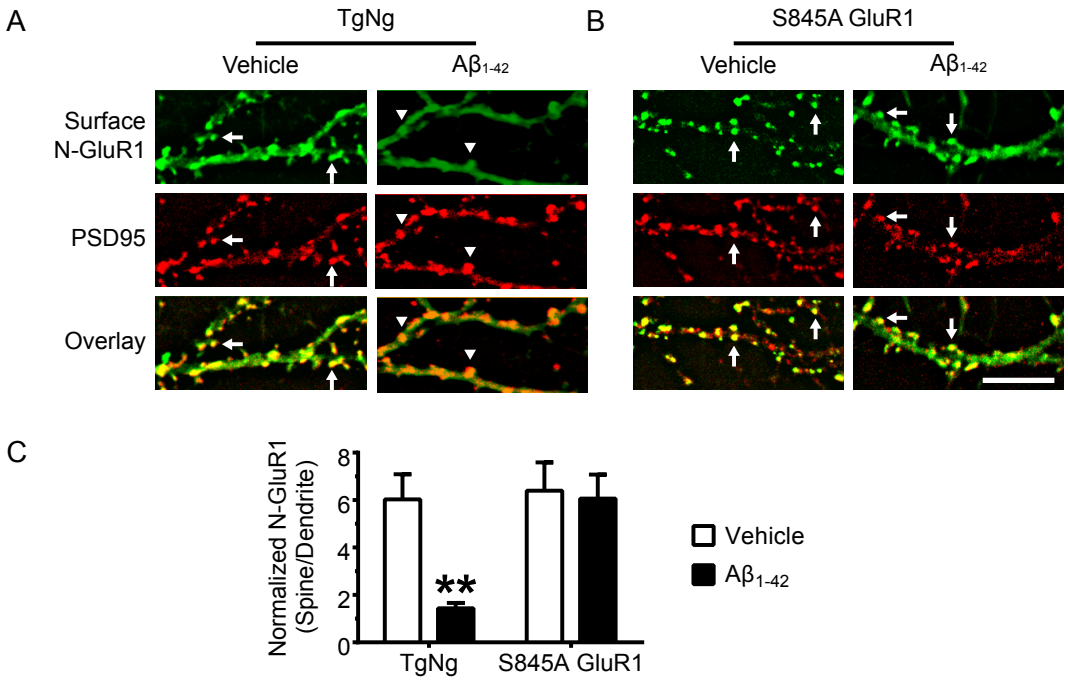

Figure 15. $A\beta_{1-42}$ treatment leads to the internalization of dendritic spine GluR1 AMPAR subunits in a manner that is dependent upon GluR1 S845 activity
A. Representative images of hippocampal neurons cultured from TgNg mice and stained with antibodies to N-GluR1 (Green) and PSD-95 (Red). At 21 DIV neurons were treated with vehicle or $A\beta_{1-42}$ and then imaged after 3 days. Arrows indicate spines with baseline N-GluR1 fluorescence, arrowheads indicate spines with lowered N-GluR1 fluorescence. Scale bar represent 10 μm.
B. Representative images of hippocampal neurons cultured from S845A GluR1 mice and stained with antibodies to N-GluR1 (Green) and PSD-95 (Red). At 21 DIV neurons were treated with vehicle or $A\beta_{1-42}$ and then imaged after 3 days.
C. Quantification of N-GluR1 expression in neurons shown in (A) and (B). Fluorescent intensity of the green channel was measured at individual dendritic spines and adjacent dendritic shafts. Spine intensity was then normalized to dendrite intensity for each spine. Two-way ANOVA, Tukey post-test used for differences between individual groups, **$p < 0.01$

III. Discussion

To better understand the role of tau mislocalization in the early stages of AD, we conducted experiments that determined soluble Aβ oligomers cause mislocalization of

tau to dendritic spines in a phosphorylation-dependent manner. We also unraveled signaling mechanisms downstream of tau in synaptic deficits induced by treatment with soluble Aβ oligomers (Figure 16). Our findings highlight the significance of tau mislocalization to dendritic spines and extend our understanding of the signaling mechanisms that mediate synaptic deficits caused by soluble Aβ oligomers in AD.

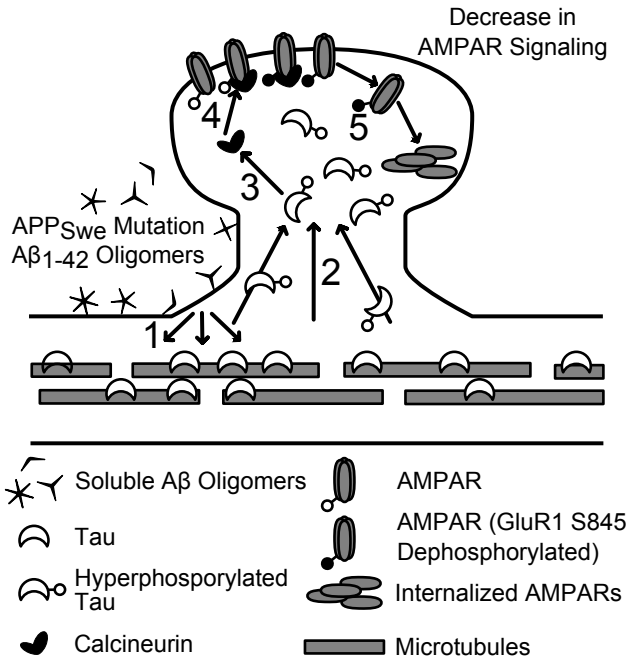

Figure 16. Proposed pathway of soluble $A\beta_{1-42}$ oligomer-induced plasticity at dendritic spines
1. Treatment with $A\beta_{1-42}$ leads to tau hyperphosphorylation. 2. Tau mislocalizes to dendritic spines. 3. Mislocalization of tau provokes activation of calcineurin. 4. Activated calcineurin dephosphorylates AMPAR GluR1 subunit residue S845. 5. AMPAR receptors are internalized.

A. The Role of Tau Mislocalization to Dendritic Spines in AD

Our groups previously reported the mislocalization of tau to dendritic spines in transgenic mice modeling frontotemporal dementia (Hoover et al., 2010). The current

findings build on previous observations that tau mislocalizes to the somatodendritic compartment of neurons in AD transgenic mice (Ittner et al., 2010; Zempel et al., 2010), however we critically advanced this notion by showing that tau mislocalizes to the dendritic spine. We found that tau_{P301L} transgenic mice exhibit cognitive deficits that correlate with the mislocalization of tau to dendritic spines (Hoover et al., 2010). The present report extends our previous findings by demonstrating that tau mislocalization occurs in the presence of increased soluble Aβ oligomers in the absence of tau mutation. We show that tau mislocalization to dendritic spines occurs in neurons from APP_{Swe} transgenic mice and in neurons exposed to synthetic oligomerized $A\beta_{1-42}$. The implications of tau presence in dendritic spines are vast as most excitatory glutamatergic neurotransmission in the brain occurs in dendritic spines (Cingolani and Goda, 2008; Patterson and Yasuda, 2011). Spines are specialized signaling structures in which protein signaling is localized by the long thin neck of the spine and the binding properties of anchoring proteins found in the spine (Bloodgood and Sabatini, 2005; Byrne et al., 2011). When tau enters a spine it is exposed to numerous proteins that serve functions in plasticity - proteins that gate the entry of Ca^{2+} into the spine, proteins that control the expression and dynamics of glutamate receptors, and numerous anchoring proteins (Lee et al., 2009; Malenka and Bear, 2004; Matsuzaki et al., 2004). We hypothesize that the entry of tau into dendritic spines underlies the early stages of the disease process in AD.

B. Phosphorylation of Tau is Critical for Oligomerized $A\beta_{1-42}$-Induced Tau Mislocalization

Although the phenomenon of tau phosphorylation in AD and other dementias has been widely reported (Avila et al., 2004; Buerger et al., 2005; Grundke-Iqbal et al., 1986; Mucke et al., 2000; Wang et al., 2013), evidence for a mechanistic role of tau phosphorylation in synaptic deficits found in AD models has been scarce (Hoover et al., 2010; Steinhilb et al., 2007). Some evidence suggests that soluble Aβ induces tau phosphorylation (Takashima et al., 1996; Wang et al., 1998). Phosphorylation of tau by proline-directed kinases regulates microtubule-binding activity of tau (Alonso et al., 1994; Wang et al., 1998). In our past exploration of tau mislocalization in neurons expressing P301L tau we were able to manipulate the phosphorylation of tau by co-expressing APtau. We showed that phosphorylation of tau at SP/TP residues is necessary for both the mislocalization of tau and decreases in AMPAR signaling (Hoover et al., 2010). In the current study we have extended these findings by showing that SP/TP phosphorylation of tau is necessary for oligomerized $A\beta_{1-42}$-induced tau mislocalization. These results expand our knowledge of the relationship between tau phosphorylation and mislocalization from frontotemporal dementia to AD. This extends our hypothesis that the entry of tau into dendritic spines is mediated by hyperphosphorylation of the protein. This highlights the aberrant phosphorylation of tau as a potential target in the treatment of AD and other dementias. Additionally, this adds further evidence of the importance of proline-directed serine/threonine kinases, like glycogen synthase kinase 3 (GSK3) (Hooper et al., 2008), in tau hyperphosporylation found in AD.

C. Calcineurin Mediates Oligomerized $A\beta_{1-42}$-Induced Synaptic Deficits via GluR1 S845

A number of research groups have reported the necessity of calcineurin in cognitive and synaptic deficits found in AD models (Abdul et al., 2009; Chen et al., 2002; Dineley et al., 2007; Snyder et al., 2005; Wu et al., 2010). Calcineurin is a phosphatase that has been widely implicated in long term depression, which shares a number of features with the synaptic deficits found in AD (Dell'Acqua et al., 2006; Miller et al., 2012; Mulkey et al., 1993). In both long term depression and models of AD, AMPAR currents and spine structure are affected (Hsieh et al., 2006; Sheng et al., 2012; Zhou et al., 2004). Here, we report that the amplitude of AMPAR currents is decreased in a calcineurin-dependent manner when neurons are treated with oligomerized $A\beta_{1-42}$. This finding is congruent with past findings demonstrating the importance of calcineurin in synaptotoxicity associated with AD; we add to this body of literature by demonstrating a role for calcineurin early in the disease process. GluR1 S845 has been shown to be dephosphorylated by calcineurin (Kam et al., 2010; Lee et al., 1998). Our finding that the blockade of activity at GluR1 S845 prevents oligomerized $A\beta_{1-42}$-induced GluR1 internalization provides a link between calcineurin and reduced AMPAR signaling. This supports our proposed model in which calcineurin acts downstream of tau in the signaling pathway mediating oligomerized $A\beta_{1-42}$-induced synaptic deficits (Figure 16).

Calcineurin is activated by the entry of low concentrations of Ca^{2+} into the cytoplasm (Dell'Acqua et al., 2006). In models of AD, it is proposed that Ca2+ homeostasis is dysregulated by modulation of NMDA glutamate receptors, L-Type Ca^{2+}

channels and/or mitochondrial Ca^{2+} buffering (Green, 2009). Ca^{2+} levels are increased in neurons exposed to elevated Aβ levels (Kuchibhotla et al., 2008; Wu et al., 2010; Zempel et al., 2010). The mislocalization of tau to dendritic spines may initiate a signaling process that leads to the entry of low levels of Ca^{2+} into the intracellular space and activation of calcineurin.

D. Functional Deficits Likely Precede Spine Loss in Oligomerized $A\beta_{1-42}$-Treated Neurons

Our results suggest that oligomerized $A\beta_{1-42}$-induced spine loss is downstream of decreases in AMPAR signaling. We do not find spine loss in neurons treated with oligomerized $A\beta_{1-42}$ at 1 or 3 days (Figure 12). After 3 days of oligomerized $A\beta_{1-42}$ treatment we see decreases in the amplitude, but not frequency, of AMPAR currents (Figure 14). Changes in mEPSC amplitude represent an alteration in the AMPAR compliment at the synapse while changes in mEPSC frequency are attributed to modifications in presynaptic release or synapse density. The lack of change in mEPSC frequency of neurons treated with oligomerized $A\beta_{1-42}$ supports our finding of no change in spine density in our live-imaging experiment. Most groups that have investigated changes in spine density after treatment with soluble Aβ oligomers have not seen them until 5 or more days after treatment (Hsieh et al., 2006; Shankar et al., 2007; 2008; Sheng et al., 2012; Shrestha et al., 2006). If this is the case, then we suspect that deficits in AMPAR signaling occur early after exposure, before spine loss, in neurons treated with soluble Aβ oligomers. Research into the mechanisms of LTD and LTP has indicated that

functional changes occur prior to structural changes (Lee et al., 2009; Matsuzaki et al., 2004; Zhou et al., 2004). In slice culture experiments in which Aβ precursor protein was overexpressed, protection against AMPAR internalization via mutation of GluR2 residue R845 also prevented spine loss (Hsieh et al., 2006). We predict that the GluR1 S845A mutation would have a similar effect and protect against spine loss in oligomerized $A\beta_{1-42}$-treated neurons as well as GluR1 internalization at late time points.

E. Conclusions

Our results implicate the mislocalization of tau to dendritic spines in neurons exposed to soluble $A\beta_{1-42}$ oligomers *in vitro*. We have also delineated signaling mechanisms that mediate oligomerized $A\beta_{1-42}$-induced synaptic deficits (Summarized in Figure 16). These findings contribute to the large body of research seeking to unravel mechanisms of synaptic deficits in AD and, more importantly, highlight the significance of tau mislocalization in the dementia. The role of tau phosphorylation and mislocalization in AD must be considered as new therapeutics are developed to combat the disease.

Chapter 4: A53T α-synuclein expression causes tau mislocalization to dendritic spines and deficits in AMPA glutamate receptor signaling via GSK3-mediated tau phosphorylation

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Authorship Contributions
Participated in research design: Miller, Lee, and Liao.
Conducted experiments: Miller, Teravskis Lee, and Liao.
Performed data analysis: Miller, Teravskis, Lee, and Liao.
Wrote or contributed to the writing of the manuscript: Miller, Teravskis, and Liao.

I. Introduction

As the second most prevalent neurodegenerative disorder, Parkinson's disease (PD) affects 1.5% of the population over 65 years of age (Alves et al., 2008; Meissner et al., 2011). PD is commonly characterized by motor dysfunction including the three hallmark symptoms: bradykinesia, rigidity and resting tremors (Mardsen et al. 1982). However, as many as 80% of PD patients also suffer from non-motor symptoms, such as depression and PD-related dementia (Aarsland et al., 2008; Reichmann et al., 2009; Svenningsson et al., 2012). Lewy bodies are found ubiquitously in PD patients and are mainly composed of the protein α-synuclein (αSyn) (Spillantini et al., 1997; 1998). The physiological role of αSyn is somewhat unclear; however, recent evidence suggests that αSyn is involved in the regulation of the synaptic vesicle recycling pool (Nemani et al., 2010), and promotes SNARE-complex assembly (Burré et al., 2010).

The autosomal dominant αSyn point mutation A53T was identified in familial strains of PD (Polymeropoulos et al., 1997). A53T expression has been shown to cause αSyn aggregation, motor deficits, neuronal loss, and impairment of neuronal function in mice (Dev et al., 2003; Lee et al., 2002). Recent evidence suggests that while the A53T mutation increases the neurotoxicity of αSyn it does not significantly inhibit the presynaptic role of αSyn in SNARE-complex assembly (Burré et al., 2012).

The microtubule-associated protein tau has been found in a hyperphosphorylated state in multiple neurodegenerative disorders including PD and Alzheimer's disease (AD) (Avila et al., 2004; Buée et al., 2000). Moreover, tau protein has been shown to

colocalize with αSyn in Lewy bodies (Ishizawa et al., 2003). Tau is normally found in the axonal compartment of neurons, but has recently been shown to invade the dendritic compartment in disease models (Avila et al., 2004; Ittner et al., 2010; Zempel et al., 2010). Our group recently discovered that tau hyperphosphorylation leads to its mislocalization in dendritic spines resulting in synaptic impairment (Hoover et al., 2010). Clinical research suggests that tau and αSyn synergistically increase PD risk (Goris et al., 2007). The phosphorylation of tau negatively affects its physiological functions, such as microtubule binding (Alonso et al., 1994; Wang et al., 1998). Hyperphosphorylation of tau has been observed in both PD and AD (Duka et al., 2009; Haggerty et al., 2011; Lei et al., 2010); additionally, the expression of the A53T mutation is coincident with the formation of filamentous tau inclusions in both mouse and human subjects (Giasson et al., 2003; Kotzbauer et al., 2004).

Little research into a possible postsynaptic role of αSyn has been conducted. Based on the implications that A53T αSyn and tau are both involved in the pathogenesis of PD we hypothesize that the two proteins may interact and cause deficits in cognitive and postsynaptic functioning. We found that A53T αSyn transgenic mice display cognitive deficits at 7 months of age. Using fluorescent microscopy, we determined that tau mislocalizes to dendritic spines in neurons cultured from A53T αSyn transgenic mice in a tau phosphorylation-dependent manner. Employing whole-cell patch-clamp electrophysiology we found that transfection of rat hippocampal neurons with a plasmid encoding A53T αSyn led to a decrease in the amplitude of miniature excitatory postsynaptic currents (mEPSC). To better understand the role of tau phosphorylation in

postsynaptic deficits caused by the A53T mutation we pharmacologically inhibited the kinase GSK3 and found that GSK3 activity is necessary for the synaptic deficits found in neurons transfected with A53T αSyn. Our results indicate that we have discovered a novel postsynaptic deficit caused by the A53T αSyn mutation that requires tau phosphorylation by GSK3 and subsequent tau mislocalization to the dendritic spine.

II. Results

A. A53T Mutation of αSyn Leads to Cognitive Deficits in Transgenic Mice

We first sought to determine if A53T αSyn transgenic mice exhibit cognitive deficits. Our collaborators in the lab of Dr. Michael Lee at the University of Minnesota tested the learning abilities of A53T transgenic mice and their transgenic negative (TgNg) littermates with the Y-maze at 7 months of age. We found that A53T mice alternated their arm visits at a significantly lower rate than TgNg mice (Figure 17A, t test, $P < 0.05$). A53T mice also exhibited a significantly lower spatial preference than TgNg mice (Figure 17B, t test, $P < 0.05$). Hippocampal damage impairs performance on these measures in the Y-maze task. Notably, no significant differences were found in the number of arm visits, indicating that motor deficiencies were not present at a time that cognitive deficits are found (Figure 17C). To confirm transgenic A53T αSyn expression we took slices of cortical tissue from A53T transgenic mice, fixed the slices, and then stained with antibodies to phospho-serine 129 αSyn, a site that is highly phosphorylated in A53T mice. We found a robust expression of A53T in the axon, soma, and dendrites

of cortical neurons (Figure 17D). These results indicate that expression of A53T αSyn in mice leads to cognitive deficits prior to the development of motor deficits.

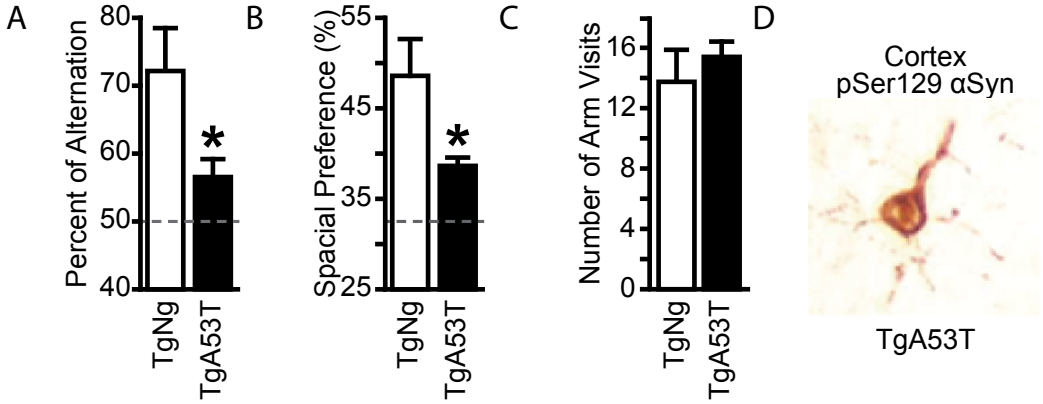

Figure 17. Impaired Hippocampal Memory in A53T αSyn Transgenic Mice
A. Mice were tested on a Y-maze alternation task. Alternation rate was measured. A53T αSyn transgenic mice performed significantly worse on the task than their TgNg littermates. Impaired performance on this task is associated with hippocampal damage.
B. The spatial preference score of mice in the Y-maze task was also measured. A53T αSyn transgenic mice significantly decreased scores compared to their TgNg littermates.
C. Number of arm visits was measured and the two groups did not perform significantly differently. This indicates that motor deficits are not responsible for the decreased performance of A53T αSyn mice on the two previous measures.
D. A slice of cortical tissue from an A53T αSyn mouse was stained with an antibody to αSyn pSer129. Phophorylated αSyn is shown present in the axon, soma, and dendrites of the neuron.
T test, *$p < 0.05$

B. A53T αSyn Causes Postsynaptic Deficits in AMPAR Signaling

After finding that A53T αSyn transgenic mice display cognitive deficits we hypothesized that A53T αSyn may cause synaptic deficits as well. To address this question we cultured dissociated rat hippocampal neurons and then transfected them with GFP, wild-type αSyn tagged with GFP (WT αSyn), or A53T αSyn tagged with GFP at 7-

10 days *in vitro* (DIV). All constructs were tagged with GFP to allow for identification of transfected neurons. At 20-23 DIV, when the neurons were mature, we applied whole-cell voltage-clamp electrophysiology techniques to transfected neurons in order to record AMPAR miniature excitatory postsynaptic currents (mEPSC) (Figure 18A). As calcium phosphate transfection only transfects ~5% of cells in the dish most axons synapsing on the neuron of interest do not express the transgene. Thus, we attribute changes in AMPAR mEPSCs to cell-autonomous effects of plasmid transfection. The amplitudes of mEPSCs were markedly smaller in neurons transfected with A53T αSyn than in neurons transfected with GFP or WT αSyn (Figure 18B; Kolmogorov-Smirnov Test, $D = 0.2139$, $P < 0.0001$). Furthermore we found that neuronal expression of A53T αSyn caused a significant decrease in average mEPSC amplitude compared to neurons transfected with GFP or WT αSyn. (Figure 18C, one-way ANOVA, $F_{2,33} = 7.093$, $P = 0.0027$; Tukey post-test used for differences between individual groups). No significant difference was found between groups in mEPSC frequency (Figure 18D). These results indicate that A53T αSyn causes a postsynaptic decrease in AMPAR signaling.

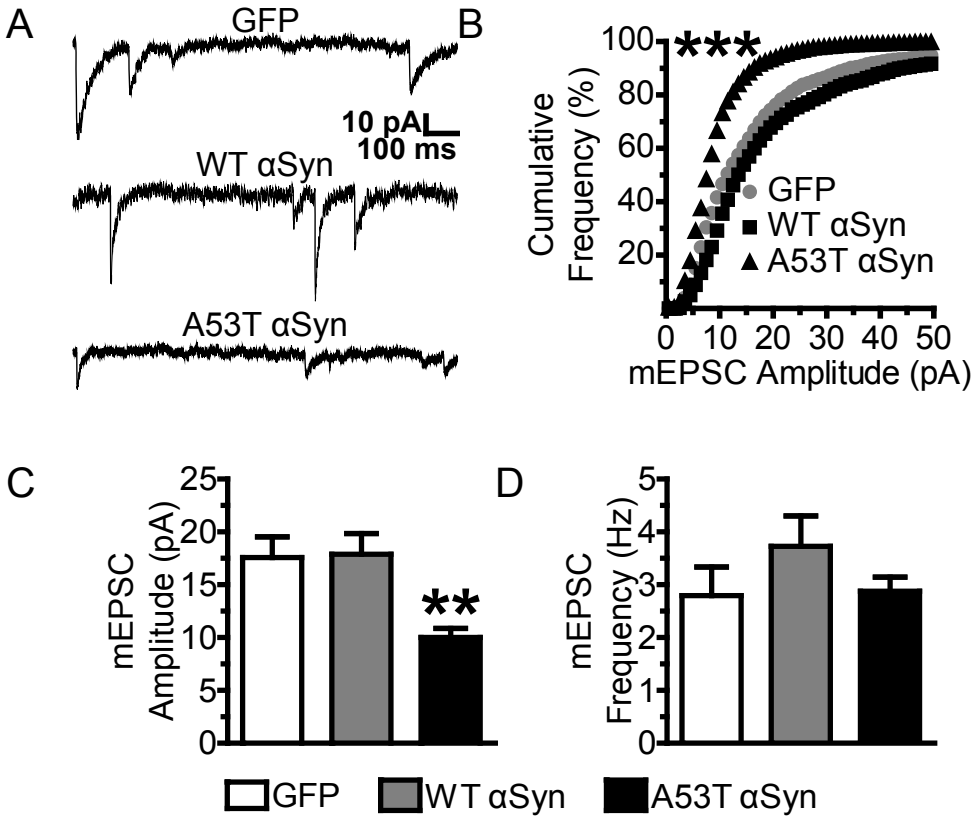

Figure 18. Expression of A53T αSyn in cultured hippocampal neurons leads to a decrease in AMPAR mEPSC amplitude

A. Representative traces of AMPAR mEPSCs recorded from cultured hippocampal neurons transfected with GFP, WT αSyn, or A53T αSyn. x-axis = 100 ms, y-axis = 10 pA.

B. Cumulative frequency distributions of mEPSC amplitudes of groups shown in (A). Neurons expressing A53T αSyn had significantly more small mEPSCs than neurons from the other two groups. Bin size = 1 pA.

C. Mean mEPSC amplitude of neurons shown in (A). Mean amplitude of mEPSCs from A53T αSyn expressing neurons was significantly lower than those from the other two groups.

D. Mean mEPSC frequency of neurons shown in (A). No significant differences were found between groups.

One-way ANOVA, Tukey post-test used for differences between individual groups, **$p < 0.01$

C. A53T αSyn Causes the Mislocalizaiton of Tau to Dendritic Spines in a Tau Phosphorylation-Dependent Manner

After discovering synaptic and cognitive deficits related to expression of A53T αSyn we hypothesized that the mutation may cause changes in tau localization similar to those found in the P301L model of frontotemporal dementia as well as the soluble oligomeric Aβ model of AD (Hoover, *et al.*, 2010; Chapter 3). To test this hypothesis and determine the role of tau phosphorylation in the mislocalization of tau to dendritic spines we cultured hippocampal neurons from A53T αSyn transgenic mice and their TgNg littermates. At 7-10 DIV we transfected the neurons with GFP-tagged tau constructs, allowing the visualization of tau localization, and DsRed, revealing neuronal morphology. The tau constructs used encoded human WT tau, APtau (in which 14 serine (s)/threonine (t) residues phosphorylated by proline (p)-directed kinases (SP/TP) are rendered nonphosphorylatable) or E14tau (an inverse of AP tau, which mimics phosphorylation). We next imaged the neurons at 21 DIV to determine the localization of tau (Figure 19A). We found that the spines of neurons from A53T αSyn mice contained significantly greater amounts of tau than those of TgNg. Tau was not highly expressed in the dendritic spines of neurons from A53T αSyn mice that were transfected with APtau. Neurons from both TgNg and A53T αSyn mice transfected with E14tau both expressed tau in dendritic spines at significantly greater levels than TgNg neurons transfected with WT tau (Figure 19B, two-way ANOVA, Interaction $F_{2,42} = 27.27$, $P < 0.0001$, Genotype $F_{2,42} = 100.2$, $P < 0.0001$, Transfection $F_{1,42} = 34.96$, $P < 0.0001$; Tukey post-test used for differences between individual groups). We found no

differences in the density of spines between groups (Figure 19C). These results indicate that the A53T mutation of αSyn causes mislocalization of tau to dendritic spines in a SP/TP phosphorylation-dependent manner. Moreover, hyperphosphorylation of tau SP/TP sites via E14tau transfection was sufficient to cause tau mislocalization, occluding the effects of A53T αSyn on tau localization.

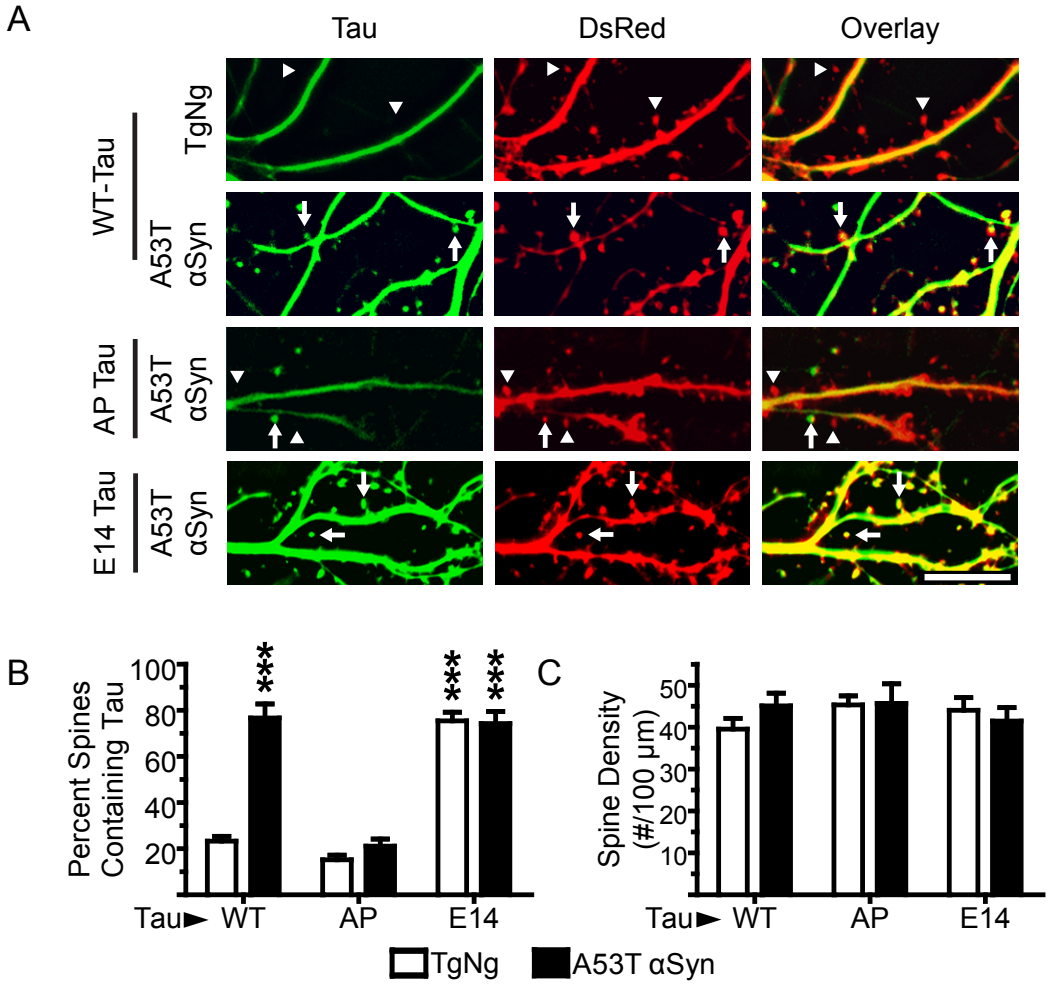

Figure 19. WT Tau is mislocalized to dendritic spines in hippocampal neurons cultured from A53T αSyn transgenic mice

A. Representative images of hippocampal neurons cultured form TgNg or A53T αSyn transgenic mice and then transfected with DsRed and either WT tau, AP tau, or E14 tau

(GFP) (TgNg neurons transfected with AP tau or E14 tau not shown). Images taken at 21 DIV. Arrows indicate spines that contain tau, arrowheads indicate spines lacking tau. Scale bar represent 10 μm.
B. Quantification of dendritic spines containing tau. WT tau is found in a significantly greater proportion of spines on neurons from A53T αSyn mice than TgNg mice. In both culture groups AP tau is not mislocalized, but E14 tau is, at a level significantly greater than TgNg mice transfected with WT tau.
C. Quantification of spine density as calculated by visual inspection of DsRed fluorescence. No significant difference was found in spine density between groups. Two-way ANOVA, Tukey post-test used for differences between individual groups, ***$p < 0.001$

D. GSK3 activity is necessary for A53T αSyn-induced tau mislocalization and synaptic depression

To further understand the role of tau phosphorylation in A53T αSyn-induced decreases in AMPAR signaling, we modulated the activity of the proline-directed serine/threonine kinase Glycogen Synthase Kinase-3 (GSK3). GSK3, which is most highly expressed in the brain as the GSK-3β isoform, has been shown to phosphorylate tau and reduce the microtubule-binding activity of tau (Lovestone et al., 1994; Wagner et al., 1996). αSyn has been shown to catalyze the phosphorylation of tau by GSK3; the three proteins have been proposed to form a heterotrimer complex (Duka et al., 2009; Haggerty et al., 2011; Kawakami et al., 2011). We first sought to determine if GSK3 activity is necessary for tau mislocalization in cells expressing A53T αSyn. We cultured neurons from A54T αSyn transgenic mice and then transfected them with WT tau tagged with GFP and DsRed. We then treated the neurons with 3 μM CHIR (a GSK3 inhibitor) or vehicle at 16 DIV and then imaged the neurons at 21 DIV (Figure 20A). We found that A53T αSyn neurons expressed tau in their spines at levels significantly higher than TgNg neurons while CHIR treatment abolished the effect (Figure 20B, one-way

ANOVA, $F_{2,21} = 46.45$, $P < 0.0001$; Tukey post-test used for differences between individual groups). No differences in spine density were found between groups (Figure 20C). These results indicate that GSK3 is necessary for mislocalization of tau to dendritic spines in neurons cultured from A53T transgenic mice.

We next hypothesized that the phosphorylation of tau by GSK3 is necessary for synaptic deficits in neurons from A53T αSyn mice. To test this hypothesis we transfected cultured rat hippocampal neurons with GFP, WT αSyn, or A53T αSyn at 7-10 DIV. We then treated neurons with CHIR at 16 DIV and recorded mEPSCs from transfected neurons 5 days later at 21 DIV using whole-cell voltage-clamp (Figure 20D). In the presence of CHIR we found no significant difference in mEPSC amplitude or frequency between groups (Figure 20F&G). We then compared the average mEPSC amplitudes of groups treated with CHIR and A53T αSyn-transfected neurons not treated with CHIR and found a significant difference (Figure 20F, one-way ANOVA, $F_{3,44} = 3.706$, $P = 0.0184$; Tukey post-test used for differences between individual groups). These results indicate that decreases in mEPSC amplitude in neurons expressing A53T αSyn require the activity of GSK3.

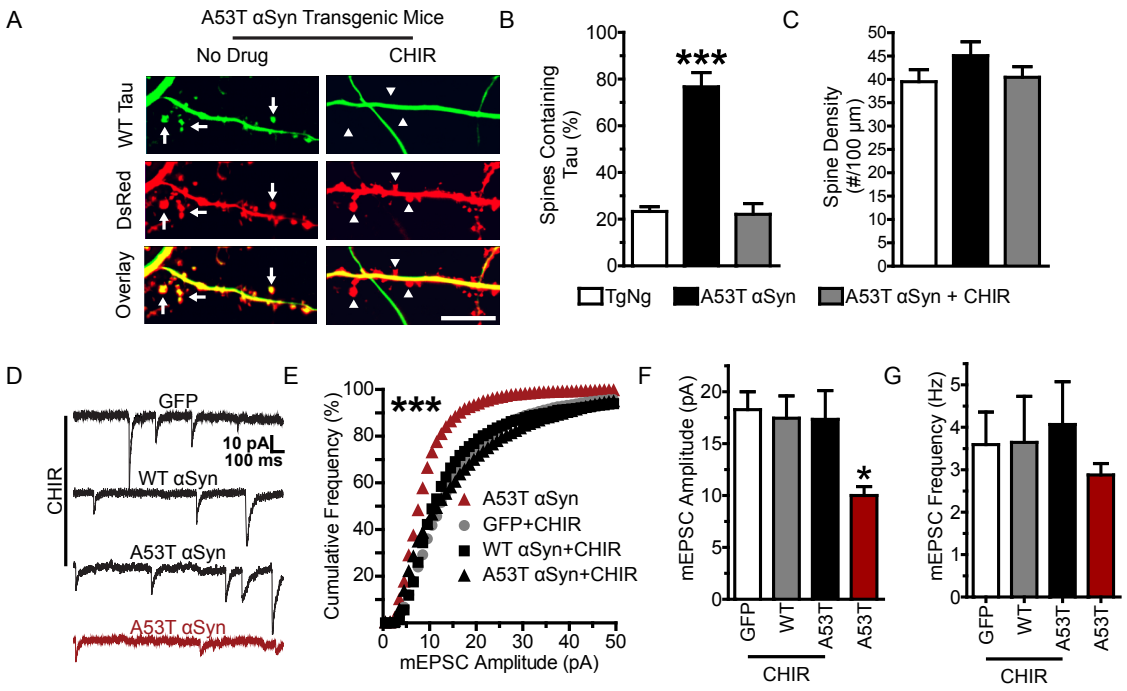

Figure 20. Activity of GSK3 is necessary for mislocalization of WT tau to dendritic spines and decreased AMPAR currents in hippocampal neurons expressing A53T αSyn

A. Representative images of hippocampal neurons cultured form A53T αSyn transgenic mice and then transfected with WT tau (GFP) and DsRed (TgNeg controls not shown). One group of A53T αSyn neurons was administered CHIR for 5 days prior to imaging at 21 DIV. Images taken at 21 DIV. Arrows indicate spines that contain tau, arrowheads indicate spines lacking tau. Scale bar represent 10 µm.

B. Quantification of dendritic spines containing tau. WT tau is found in a significantly greater proportion of spines on neurons from A53T αSyn mice than TgNg mice. A53T αSyn neurons treatmed with CHIR do not express tau in spines at a level higher than TgNg controls.

C. Quantification of spine density as calculated by visual inspection of DsRed fluorescence. No significant difference was found in spine density between groups.

D. Representative traces of AMPAR mEPSCs recorded from cultured hippocampal neurons transfected with GFP, WT αSyn, or A53T αSyn and then treated with CHIR 5 days prior to recording (A53T neurons not treated with CHIR from Figure 2 are shown for comparison). x-axis = 100 ms, y-axis = 10 pA.

E. Cumulative frequency distributions of mEPSC amplitudes of groups shown in (A). Neurons expressing A53T αSyn had significantly more small mEPSCs than neurons treated with CHIR. Bin size = 1 pA.

F. Mean mEPSC amplitude of neurons shown in (A). Mean amplitude of mEPSCs from A53T αSyn expressing neurons was significantly lower than those from groups treated with CHIR.

G. Mean mEPSC frequency of neurons shown in (A). No significant differences were found between groups.

One-way ANOVA, Tukey post-test used for differences between individual groups, ***p < 0.001, *p < 0.05

To corroborate these results we cultured hippocampal neurons from A53T αSyn and TgNg mice, treated with CHIR or vehicle at 16 DIV, and then fixed the neurons at 21 DIV. We first stained the neurons with an antibody to N-GluR1 (which only tags the extracellular N-Terminus of the GluR1 AMPAR subunit). We next permeabilized the fixed neurons and stained with an antibody to PSD-95 to reveal the locations of dendritic spines (Figure 21). We found that the expression of GluR1 in the membrane was significantly decreased in neurons cultured from A53T αSyn mice. Treatment of CHIR in A53T αSyn prevented GluR1 internalization (Figure 21B, two-way ANOVA, Interaction $F_{1,28} = 5.685$, $P = 0.0241$, Treatment $F_{1,28} = 7.182$, $P = 0.0122$, Genotype $F_{1,28} = 9.841$, $P = 0.0040$; Tukey post-test used for differences between individual groups). These results suggest that the A53T mutation of αSyn causes decreases in AMPAR signaling via postsynaptic internalization of GluR1 subunits in a manner that is dependent on the activity of GSK3.

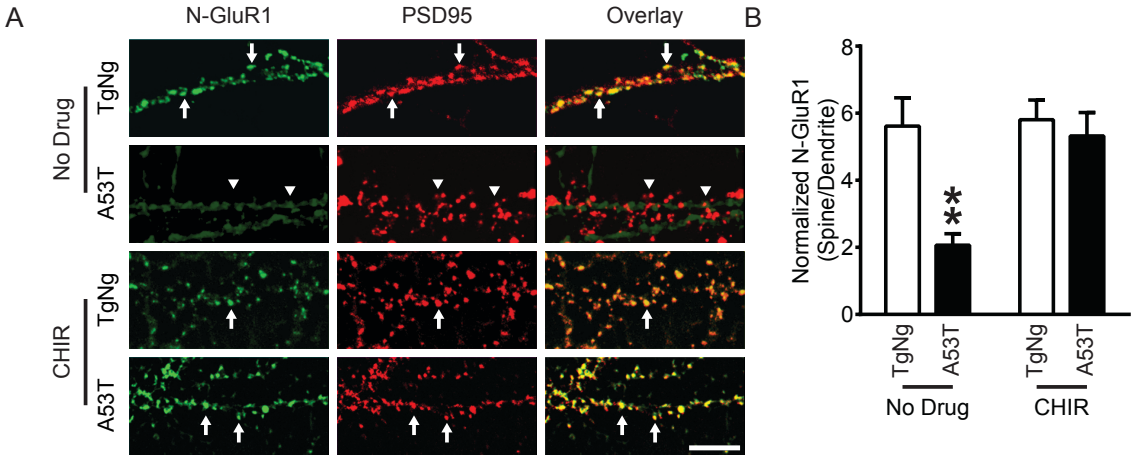

Figure 21. Internalization of dendritic spine GluR1 AMPAR subunits in A53T αSyn Transgenic Mice

A. Representative images of hippocampal neurons cultured from TgNg or A53T αSyn transgenic mice and stained with antibodies to N-GluR1 (Green) and PSD-95 (Red). At 16 DIV neurons were treated with vehicle or CHIR and then imaged 5 days later. Arrows indicate spines with baseline N-GluR1 fluorescence, arrowheads indicate spines with lowered N-GluR1 fluorescence. Scale bar represent 10 μm.

B. Quantification of N-GluR1 expression in neurons shown in (A) and (B). Fluorescent intensity of the green channel was measured at individual dendritic spines and adjacent dendritic shafts. Spine intensity was then normalized to dendrite intensity for each spine. Two-way ANOVA, Tukey post-test used for differences between individual groups, **p < 0.01

III. Discussion

The A53T point mutation of αSyn is an autosomal dominant point mutation found in familial PD (Polymeropoulos et al., 1997). Motor deficits, cell loss, and αSyn aggregation are found in transgenic mice expressing A53T αSyn (Dev et al., 2003; Lee et al., 2002). In addition to the characteristic motor disturbances found in PD, many

patients experience PD-related dementia (Aarsland et al., 2008; Reichmann et al., 2009; Svenningsson et al., 2012). We hypothesized that the A53T αSyn mutation may cause synaptic deficits that underlie PD-related dementia. We found that expression of A53T αSyn impairs learning *in vivo* and synaptic signaling *in vitro*. In light of findings implicating tau in PD (Duka et al., 2009; Goris et al., 2007; Haggerty et al., 2011; Volpicelli-Daley et al., 2011) and our group's recent finding that mislocalization of tau to dendritic spines impairs synaptic signaling (Hoover et al., 2010), we also investigated the involvement of tau in neurons expressing A53T αSyn. We found that A53T αSyn-induced synaptic deficits are dependent upon GSK3-mediated tau phosphorylation and mislocalization to the dendritic spine (Figure 22). Our findings expose a novel postsynaptic role of A53T αSyn in PD-related dementia.

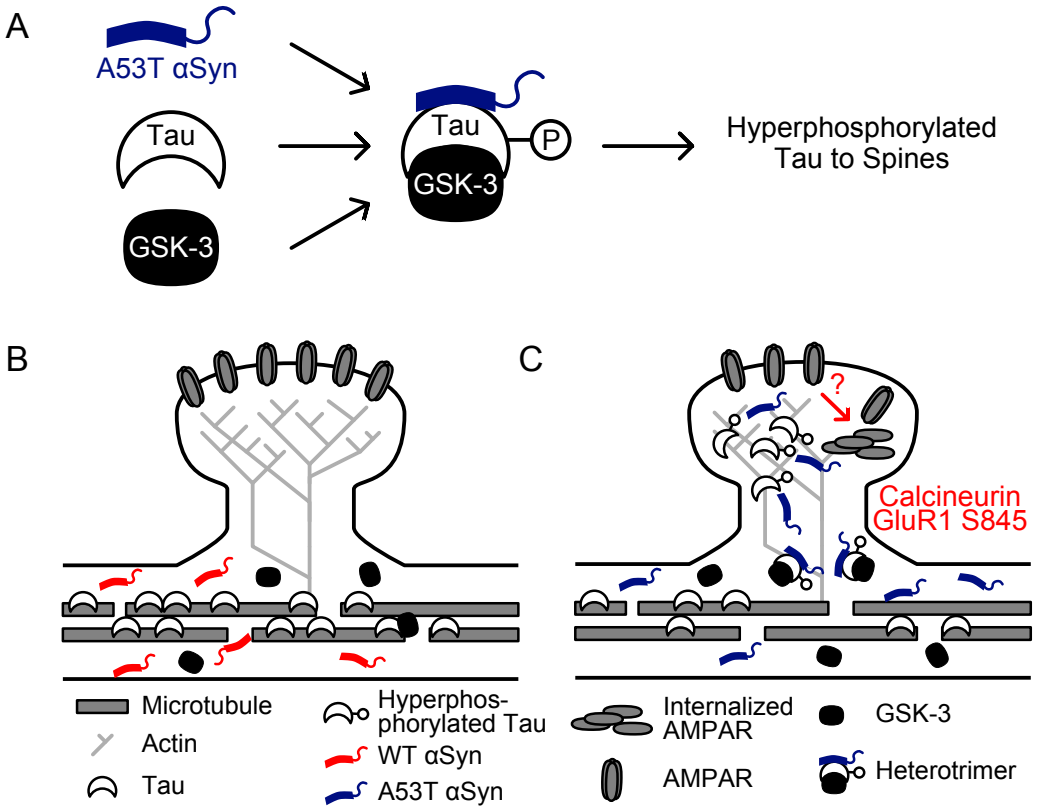

Figure 22. Proposed pathway of A53T αSyn–induced plasticity at dendritic spines
A. Hypothetical model of how A53T αSyn may form a heterotrimer with GSK3 and tau promoting tau hyperphosphorylation.
B. A normal neuron in which WT αSyn is expressed. Tau is not hyperphosphorylated and mislocalized to the dendritic spine.
C. A diseased neuron in which A53T is expressed. Tau is hyperphosphorylated and found in the dendritic spine. AMPARs are internalized in a process that may be similar to that found in Chapter 3.

A. A Novel Postsynaptic Role of A53T αSyn in Synaptic Deficits

Research into the physiological role of αSyn has indicated that the protein serves presynaptic functions (Burré et al., 2010; Nemani et al., 2010). Little research has investigated the potential postsynaptic role of αSyn. Thus, we were surprised to find that transfection of neurons with A53T αSyn impaired AMPAR signaling in a postsynaptic

manner (Figure 18). Our findings demonstrate that neurons transfected with A53T αSyn have decreased AMPAR mEPSC amplitude when compared with control groups (Figure 2). Changes in mEPSC amplitude are attributed to variations in the AMPAR compliment or kinetics at the synapse. Furthermore, our transfection method expresses plasmids in ~5% of cultured neurons, implying that changes in signaling found in transfected neurons are due to cell-autonomous effects. These factors indicate that the A53T αSyn-induced synaptic deficits that we have identified in this report are mediated by postsynaptic mechanisms. Although groups have found that overexpression of WT αSyn is sufficient to cause deficits in presynaptic function (Nemani et al., 2010), our results did not reveal any detrimental postsynaptic effects of WT αSyn expression. These results indicate that the A53T mutation of αSyn specifically causes synaptic deficits in a novel postsynaptic manner.

B. Tau Mislocalization to Dendritic Spines Occurs in Correlation with Synaptic Deficits in Multiple Neurodegenerative Diseases

In past reports, our group has demonstrated that tau mislocalization to dendritic spines and decreased AMPAR currents are induced in models of frontotemporal dementia (Hoover et al., 2010) and AD (Chapter 3). Furthermore, we demonstrated that SP/TP phosphorylation of tau is critical for mislocalization to dendritic spines in both cases (Hoover, *et al.*, 2010; Chapter 3). In the current report we extend these findings to PD by showing tau mislocalization in a phosphorylation-dependent manner in cells cultured from A53T transgenic mice (Figure 19). Tau mislocalization to dendritic spines warrants

further investigation. Dendritic spines are the site of most excitatory glutamatergic neurotransmission in the brain (Cingolani & Goda, 2008; Patterson & Yasuda, 2011). Ionic and protein signaling is localized to the spine by its long thin neck and the binding properties of anchoring proteins found in the spine (Bloodgood & Sabatini, 2005; Byrne et al., 2011). Numerous anchoring proteins, proteins that control the expression and dynamics of glutamate receptors, and proteins that gate the entry of Ca2+ into the spine greet tau as it enters the dendritic spine (Malenka & Bear, 2004; Matsuzaki et al., 2004; Lee et al., 2009). We are unsure of the mechanisms underlying A53T αSyn-induced detrimental synaptic effects of tau mislocalization to dendritic spines. Our research into $A\beta_{1\text{-}42}$-induced synaptic deficits indicates that they may be mediated by calcineurin-induced dephosphorylation of AMPAR GluR1 subunit residue S845 downstream of tau (Chapter 3). Given the similarities seen between the effects of A53T αSyn and $A\beta_{1\text{-}42}$ on the localization of tau and AMPAR signaling, we hypothesize that similar mechanisms mediate signaling deficits downstream of tau in both phenomena.

C. GSK3-Mediated Phosphorylation of Tau Is Critical for Both Tau Mislocalization and Synaptic Deficits Caused by Expression of A53T αSyn

GSK-3 plays a diverse range of physiological roles in development, including cellular division, proliferation, differentiation and adhesion (Embi et al., 1980; Lei et al., 2011; Yao et al., 2002). Recent evidence has also implicated GSK3 in synaptic plasticity, particularly long-term depression of synapses (Bradley et al., 2012). GSK3 is known to phosphorylate tau and is thought to play a role in tau-mediated neurodegeneration (Lei et

al., 2011). GSK3 phosphorylation is elevated in the brains of PD patients and recent studies indicate that αSyn stimulates the hyperphosphorylation of Tau by GSK-3β (Duka et al., 2009; Haggerty et al., 2011; Kawakami et al., 2011; Lei et al., 2010). Furthermore, A53T αSyn is sufficient to cause increased GSK3 and tau phosphorylation (Wills et al., 2011). We found that GSK3 activity is necessary for tau mislocalization to dendritic spines, decreases in AMPAR signaling, and internalization of AMPAR subunit GluR1 in cells expressing A53T αSyn (Figures 20 & 21). These findings further implicate GSK3 in PD and support our findings implicating SP/TP tau phosphorylation in tau mislocalization. Additionally, the finding that A53T αSyn-induced tau mislocalization and synaptic deficits are both abrogated by GSK inhibition indicates that the processes are linked and adds credence to our hypothesis that synaptic deficits are downstream of tau mislocalization in our study.

D. Further *In Vivo* Research Must Be Conducted to Determine if Learning Impairments Correlate With Impairment of Plasticity Induction

PD-related dementia is an auxillary symptom found in a large number of those with PD (Aarsland et al., 2008; Reichmann et al., 2009; Svenningsson et al., 2012). We found cognitive deficits at 7 months of age in A53T αSyn transgenic mice (Figure 1). Deficits in long-term potentiation have long been correlated with impaired learning behavior in AD models (Chapman et al., 1999; Gureviciene et al., 2004; Hoover et al., 2010). We hypothesize that long-term potentiation would be impaired in these transgenic mice at 7 months of age. If that were the case, it would be important to determine if

GSK3-mediated phosphorylation and tau mislocalization are necessary for hypothesized deficits in long-term potentiation and our observed cognitive deficits in A53T αSyn transgenic mice. Further research must be conducted to test these hypotheses.

E. Conclusions

The cellular and molecular mechanisms of memory loss in PD were previously unknown. This report unveils cognitive deficits in A53T αSyn transgenic mice, indicating the model's potential for study of PD-related dementia. We describe a novel postsynaptic role of αSyn, a presynaptic protein, in AMPAR signaling deficits caused by the A53T mutation of αSyn (Figure 22). Most interestingly, our results draw intriguing parallels between PD, frontotemporal dementia and AD. By expressing A53T αSyn in neurons we have found a mislocalization of tau to dendritic spines that is similar to that seen in AD models (Hoover, *et al.*, 2010; Chapter 3). Furthermore, deficits in AMPAR signaling are correlated with tau mislocalization to dendritic spines in the current study and past reports (Hoover, *et al.*, 2010; Chapter 3). This pathway may represent the early mechanisms underlying memory loss in PD (Figure 23). Moreover, these findings suggest that early synaptic deficits in multiple neurodegenerative diseases are mediated by aberrant localization of tau to dendritic spines, providing a common therapeutic target for clinicians in the treatment of dementias.

Chapter 5: Conclusions

In this dissertation I have investigated AMPAR trafficking in three different disease models. AMPAR receptor currents are decreased in cultured hippocampal neurons exposed to morphine, soluble Aβ oligomers, and A53T αSyn. In the previous chapters I further investigated the signaling pathways responsible for each type of plasticity. In Chapter 2, I showed that functional and structural plasticity caused by morphine treatment are mediated by a divergent signaling pathway involving calcineurin and CaMKII (Figure 10). In Chapter 3, I demonstrated that soluble Aβ oligomers cause the mislocalization of tau to dendritic spines and subsequent AMPAR plasticity requires the dephosphorylation of GluR1 S845 by calcineurin (Figure 16). In Chapter 4 I found that A53T αSyn causes decreases in AMPAR signaling in a manner that is dependent upon the phosphorylation and mislocalization of tau to dendritic spines (Figure 22). These three findings advance the knowledge of the signaling pathways that mediate plasticity in neurological diseases.

I. Divergent Signaling in Morphine-Induced Plasticity

The work in chapter 2 has extended the concept that divergent signaling pathways may mediate structural and functional plasticity of excitatory synapses to research on addiction. Using pharmacological and genetic interventions, we delineated the roles of calcineurin and CaMKII in functional and structural plasticity. We found that calcineurin is necessary for both types of plasticity, while CaMKII is only necessary for structural plasticity. Persistent structural and functional changes in dendritic spines caused by

drugs of abuse are proposed to mediate the aberrant learning associated with addiction (Hyman et al., 2006). Recent studies have attempted to determine the roles of synaptic plasticity in addictive drug-induced behavioral changes by either blocking cocaine-induced structural plasticity (Pulipparacharuvil et al., 2008), or cocaine-induced changes in glutamate receptor function (Moussawi et al., 2009). These behavioral studies often yield conflicting results (Moussawi et al., 2009; Pulipparacharuvil et al., 2008), which may result from divergent intracellular mechanisms underlying structural and functional plasticity of excitatory synapses caused by drugs of abuse. My thesis research provides direct experimental evidence that these two forms of drug-induced plasticity can be mediated by separate, but interacting, signaling pathways. The new knowledge gained from our *in vitro* cellular studies provide a possible explanation for conflicting results of behavioral studies that attempt to reverse only functional or only structural plasticity.

II. Tau Mislocalization to Dendritic Spines and Calcineurin-Mediated AMPAR Internalization in Neurons Exposed to Soluble Aβ Oligomers

The findings presented in Chapter 3 extend our knowledge of the importance of tau mislocalization to dendritic spines in neurodegenerative diseases and further our understanding of the signaling pathway that mediates soluble Aβ oligomer-induced AMPAR plasticity. Using fluorescently tagged tau, we demonstrated that soluble Aβ oligomers cause the mislocalization of tau to dendritic spines in a phosphorylation-dependent manner. Using a pharmacological approach, we demonstrated the necessity of

calcineurin in AMPAR signaling deficits induced by treatment of cultured hippocampal neurons with oligomerized $A\beta_{1-42}$. Using a genetic approach, we demonstrated that phosphorylation activity at GluR1 AMPAR subunit S845 is necessary for oligomerized $A\beta_{1-42}$-induced GluR1 internalization. These results broaden our groups' previous finding, that tau mislocalizes to dendritic spines in a model of frontotemporal dementia (Hoover et al., 2010), to an *in vitro* model of AD. It must be noted that the *in vitro* nature of these results limits our conclusions. These findings must be corroborated *in vivo* – in transgenic mice and humans. Our previous findings showed that tau mislocalizes to dendritic spines *in vivo* in tau_{P301L} transgenic mice (Hoover et al., 2010), future research should determine if the same effect is found in APP_{Swe} transgenic mice. Also, experiments showing the necessity of tau mislocalization to dendritic spines for behavioral deficits in mice must be conducted. These experiments would require the creation of novel tau transgenic mice expressing AP tau or another mutation of tau that prevents mislocalization *in vitro*.

III. The Role of Tau Mislocalization to Dendritic Spines in AMPAR Signaling Deficits Found in Neurons Expressing A53T αSyn

The findings in Chapter 3 uncovered novel deficits in AMPAR signaling in neurons expressing A53T αSyn that require the hyperphosphorylation and mislocalization of tau to dendritic spines. We first demonstrated the presence of cognitive deficits in A53T αS transgenic mice performing a task that requires the

hippocampus. We next cultured neurons from A53T αSyn transgenic mice and manipulated tau genetically and GSK3 pharmacologically. We showed that phosphorylation of tau is necessary for the mislocalization of tau to dendritic spine in neurons expressing A53T αS. By pharmacologically inhibiting GSK3 we demonstrated that tau phosphorylation by GSK3 is necessary for decreased AMPAR signaling in neurons expressing A53T αS. Subsequent work must be conducted to determine if these results are recapitulated *in vivo*. Additionally, the link between the cognitive deficits and *in vitro* AMPAR signaling deficits that we reported is tenuous. Investigation of LTP in slices from seven month old A53T αSyn mice could provide a stronger cellular correlate to the cognitive deficits we reported. Our results indicate that tau mislocalization to dendritic spines occurs in models of frontotemporal dementia, AD, and PD. In all three models tau mislocalization correlates with decreased AMPAR signaling and our experiments indicate an important role of tau mislocalization in this plasticity (Figure 23).

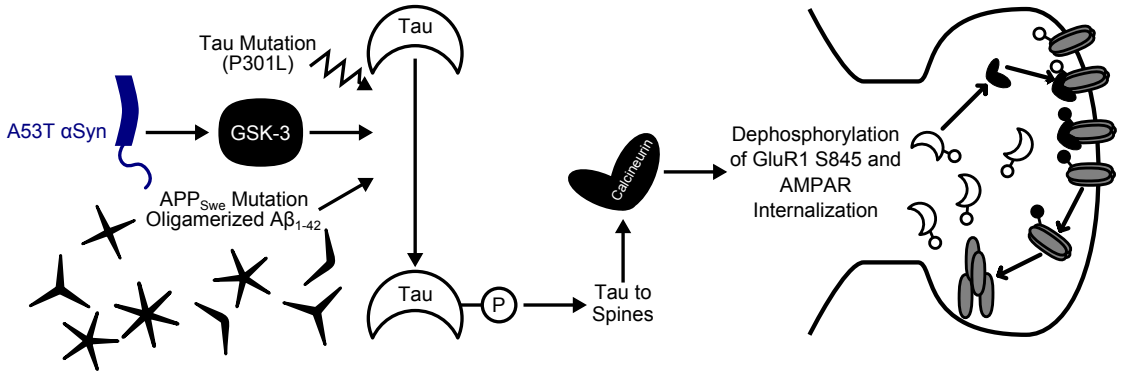

Figure 23. Proposed pathway showing the meditating role of tau mislocalization to dendritic spines in synaptic deficits found in multiple dementias
Mutation of tau, mutation of αSyn, and increased levels of soluble Aβ oligomers promote the hyperphosphorylation and mislocalization to dendritic spines of tau. This leads to

increased calcineurin-mediated GluR1 S845 dephosphorylation and internalization of AMPARs.

IV. A Unified Theory of the Role of Tau in Dementia

The understanding of Alzheimer's disease has advanced greatly over the last two decades as the research focus has repositioned to understanding the roles of soluble Aβ and tau at the synapse. Researchers have discovered that the hallmark pathologies of AD – neuritic plaques and neurofibrillary tangles – are not well correlated with dementia, indeed, soluble forms of their main constituents – Aβ and tau – are strongly correlated with dementia. The fields' focus has moved from the cell death originally identified in postmortem analysis of the brains of AD patients to delineating the synaptic deficits that have been discovered. The creation of a number of mouse models that mimic various features of the disease has allowed us to better understand the synaptic changes that occur in AD. Our current understanding of the research indicates that Aβ initiates the onset of AD while tau mediates the symptom the disease. The mislocalization of tau to the dendritic compartment and spines of neurons has been shown to be an important step leading to disruptions at synapses. The use of soluble Aβ oligomers has allowed greater study of the signaling mechanisms involved and led to the identification of a number of proteins that may be responsible for deficits.

The findings of this thesis suggest that we can expand our proposed model, positing tau as mediator of synaptic and cognitive deficits, to other forms of dementia (Figure 23). Abnormal phosphorylation of tau is found in a number of dementias besides

AD – frontotemporal dementia, Parkinson's disease, corticobasal degeneration, and Pick's disease, among others. The mislocalization of tau has been correlated with synaptic and cognitive deficits in both models of AD (Ittner et al., 2010; Zempel et al., 2010) and a model of frontotemporal dementia (Hoover et al., 2010). This thesis extends those findings by showing mislocalization of tau *to dendritic spines* in AD and PD. Our results, which implicate the phosphorylation and mislocalization of tau, may represent the identification of a common pathway for dementias (Figure 23). If this hypothesis proves correct it is possible that interventions focused on abrogating the neurotoxic activity of tau in AD could be applied to these other dementias as well.

Chapter 6: Materials & Methods

High-Density Neuronal Cultures and Neuronal Transfection

A 25-mm glass polylysine-coated coverslip (thickness, 0.08mm) was glued to the bottom of a 35-mm culture dish with a 22-mm hole using silicone sealant as previously described (Wiens et al., 2005). Dissociated neuronal cultures from rat hippocampus at postnatal days 1–2 were prepared as previously described (Liao et al., 2001). Neurons were plated onto prepared 35-mm culture dishes at a density of $1x10^6$ cells per dish. The age of cultured neurons was counted from the day of plating as 1 day *in vitro* (DIV). All experiments were performed on neurons from at least 3 independent cultures. Neurons at 7–10 DIV were transfected with appropriate plasmids using the standard calcium phosphate precipitation method as previously described (Wiens et al., 2005). After transfection, neurons were put back to a tissue culture incubator (37°C, 5% CO2) and allowed to mature and develop until three weeks *in vitro*, a time at which neurons express high numbers of dendritic spines with mature morphologies.

Low-Density Neuronal Cultures

To detect the distribution of endogenous synaptic proteins with high resolution, low-density neuronal cultures were prepared as previously described with some modifications (Hoover et al., 2010). Dissociated neuronal cultures from rat hippocampus at postnatal days 1–2 were plated into 12-well culture plates at a density of 50,000–100,000 cells per well. Each well contained a polylysine-coated 12-mm glass coverslip on the bottom. To maintain the low-density cultures for a long time (up to 1 month), the above 12-mm coverslips with low-density cultured neurons were transferred to 60 mm dishes (4 coverslips per dish; the coverslips faced up) that contained high-density neuronal cultures after 1 week *in vitro*. In previous studies, dishes with a glial feed layer were often used to support low-density cultures. Recently, we have found that high-density neuronal cultures are far better supporters than pure glial cells.

Neuronal Mouse Cultures

Following the protocol described in Strasser et al. (2004), hippocampal cultures were prepared from APP_{Swe} and A53T αSyn transgenic mice, respectively (Hsiao et al., 1996; Lee et al., 2002). For primary hippocampal neuron cultures, approximately 1.5 x 10^4 cells were plated on sets of 5 x 12 mm coverslips that had been previously coated with Poly-D-Lysine (100 mg/ml) + laminin (4 mg/ml) in neuronal plating media (MEM with Earle's salts, 10 mM HEPES, 10 mM sodium pyruvate, 0.5 mM glutamine, 12.5 mM glutamate, 10% FBS, and 0.6% glucose). Each set of 5 coverslips was maintained in a 35 mm dish and each dish corresponded to 1 mouse. Approximately 4 hr after plating, the media was replaced with either neuronal growth medium (Neurobasal media with B27 supplement, 0.5 mM glutamine) that had been conditioned on glia for 24–48 hr immediately prior to use. Mice were genotyped by PCR analysis of tail snip lysates using transgene-specific primers.

Electrophysiology

Miniature excitatory post-synaptic currents (mEPSC) were recorded from cultured dissociated rat hippocampal neurons at 21–25 DIV with a glass pipette (resistance of ~5 MΩ) at holding potentials of -55 mV and filtered at 1 kHz as previously described (Liao et al., 2005). Input and series resistances were checked before and after the recording of mEPSCs, which lasted 5–20 min. There were no significant difference in the series resistances and input resistance among various groups of experiments. One recording sweep lasting 200 ms was sampled every 1 s. Neurons were bathed in artificial cerebrospinal fluid (ACSF) at room temperature (25°C) with 100 μM APV (an NMDAR antagonist), 1 μM TTX (a sodium channel blocker), and 100 μM picrotoxin (GABAa receptor antagonist), gassed with 95% O_2–5% CO_2. The ACSF contained (in mM) 119 NaCl, 2.5 KCl, 5.0 $CaCl_2$, 2.5 $MgCl_2$, 26.2 $NaHCO_3$, 1

NaH_2PO_4, and 11 glucose. The internal solution in the patch pipette contained (in mM) 100 cesium gluconate, 0.2 EGTA, 0.5 $MgCl_2$, 2 ATP, 0.3 GTP, and 40 HEPES (pH 7.2 with CsOH). All mEPSCs were analyzed with the MiniAnalysis program designed by Synaptosoft Inc. Detection criterion for mEPSCs was set as the peak amplitude 3 pA. Each mEPSC event was visually inspected and only events with a distinctly fast-rising phase and a slow-decaying phase were accepted. The frequency and amplitude of all accepted mEPSCs were directly read out using the analysis function in the MiniAnalysis program. The averaged parameters from each neuron were treated as single samples in any further statistical analyses.

Time-Lapse Live Imaging Method

To label dendrites, high-density neurons at 7–10 DIV were transfected with plasmids encoding enhanced green fluorescence (GFP), GFP-tagged molecules, and/or DsRed. The 35-mm culture dishes fit tightly in a homemade holding chamber on a fixed platform above an inverted Nikon microscope sitting on an X–Y translation stage (Burleigh Inc.). A 60x oil lens was used for all imaging experiments. Original images were 157.3 μm wide (x-axis) and 117.5 μm tall (y-axis). The z-axis was composed of 15 images, taken at .5 μm intervals. The location of any neuron of interest was recorded by the reading of the X–Y translation stage. The culture dish was immediately put back into a tissue culture incubator after each observation. Neurons could be found again in the next observation using the X–Y translation stage (accuracy, 4 μm).

Immunocytochemistry in Fixed Tissues

Cultured neurons were fixed and permeabilized successively with 4% paraformaldehyde, 100% methanol, and 0.2% Triton X-100 (Hoover et al., 2010). For all immunocytochemistry primary and secondary antibodies, a dilution factor of 1:50 in 10% donkey serum in PBS was used. Commercial antibodies against PSD95 were used as a postsynaptic marker (rabbit

polyclonal, Invitrogen; mouse monoclonal, Millipore)(Liao et al., 2001). The rabbit polyclonal antibodies against the C terminus of GluR1 or Glu2/3 subunits were generous gifts from Dr. Richard Huganir at the Johns Hopkins University Medical School. Antisera against MOR were produced against a synthesized 15-residue peptide (NHQLENLEAETAPLP) corresponding to amino acids 384–398 (Arvidsson et al., 1995). A commercial antibody against CaMKII was used (mouse monoclonal; Invitrogen). Finally, FITC (green) or rhodamine (red)-conjugated secondary antibodies (Jackson ImmunoResearch) were used to recognize these primary antibodies.

To estimate the amount of glutamate receptors in dendritic spines, fixed rat neurons immunoreactive for PSD95 (mouse monoclonal, Millipore) and a GluR antibody (GluR1 or GluR2/3) were photographed and processed with MetaMorph software as previously described (Hoover et al., 2010). Then, immunoreactive clusters of PSD95 were autoselected using the Meta-Morph software and the location of these clusters was transferred to images displaying glutamate receptor immunoreactivity on the same neuron. PSD95 immunoreactivity was used to identify dendritic spines. A cursor was placed in the center of the glutamate receptor clusters in dendritic spines to estimate glutamate receptor immunoreactivity as fluorescent pixel intensity in the spines (value Y1). Another cursor was placed in an adjacent dendritic shaft to measure glutamate receptor fluorescent pixel intensity (value Y2) and the ratio of glutamate receptor immunoreactive fluorescence intensity in spines/dendrites (Y1/Y2) was plotted on the y-axis.

Western Blots

Western blot experiments were performed as described previously (Wiens et al., 2005). Briefly, cultured neurons were lysed in modified radioimmunoprecipitation assay buffer on ice and then harvested with a cell scraper. The same amount of protein was loaded in each lane. To determine CaMKII phosphorylation activity, cell lysates were ran on the western blot and then stained with a phospho-CaMKII antibody and then a CaMKII antibody (Genescript). To

determine Rac1 activity, a Rac1 activation kit (Upstate) was used. Cell lysates were immunoprecipitated with Rac/cdc42 Assay Reagent (PAK-1 PBD, agarose) to isolate active Rac1. Immunoprecipitated lysates and control lysates were run on a gel and then stained with a Rac1 antibody (Mouse monoclonal, Upstate). Rac1 proteins in total cell lysates were loaded as controls. Rac1 activation was quantified with ImageQuant TL (GE healthcare) and measured as a proportion of active Rac1 to total Rac1 (Li et al., 2002).

Image Analysis

Time-lapse live images from the same neuron at 21 DIV were taken before and at various time points after drug treatments as previously described (Liao et al., 2005). All digital images were analyzed with MetaMorph Imaging System (Universal Imaging Co.). Unless stated otherwise, all images of live neurons were taken as stacks and were averaged into one image before further analysis. In addition to simple averaging, stacks of images were also processed by deconvolution of nearest planes using MetaMorph. A stack of deconvolved images was then averaged into one single image. A dendritic protrusion with an expanded head that was 50% wider than its neck was defined as a spine. The number of spines from a dendrite was manually counted and normalized per 100 μM dendritic length; only dendrites with 50 μM or more of analyzable dendritic shaft were counted. The number of protrusions from a dendrite were manually counted and included both spines and non-spine protrusions. Student's t-tests were used for comparison between parameters from two groups whereas ANOVA tests were used for comparison between parameters from multiple groups (n=number of neurons; $p<0.05$, significant). All data are reported as mean ± standard error. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Constructs, and Pharmacological Inhibitors

CaMKII wild-type (WT) and CaMKII dominant-negative (DN) constructs (generous gifts from Dr. Richard Huganir at Johns Hopkins University) were tagged with enhanced GFP (referred to as GFP) on the N terminus and expressed in the pRK5 vector and driven by a cytomegalovirus (CMV) promoter (Clontech, Inc.). Rac1N17 (dominant negative; referred to as Rac-), or Rac1V12 (constitutively active; referred to as Rac+) were tagged with enhanced GFP and expressed in the pRK5 vector and driven by a CMV promoter. The Rac1 constructs have been characterized and used previously (Li et al., 2002; Wiens et al., 2005). The GFP and DsRed constructs (Clontech, Inc.) were also expressed in the pRK5 vector and driven by a CMV promoter.

All tau constructs were tagged with enhanced GFP (referred to as GFP) on the N terminus and expressed in the pRK5 vector and driven by a cytomegalovirus (CMV) promoter (Clontech, Inc.). The GFP and DsRed constructs (Clontech, Inc.) were also expressed in the pRK5 vector and driven by a CMV promoter. The WT htau construct encoded human four-repeat tau lacking the N-terminal sequences (4R0N) and contained exons 1, 4 and 5, 7, and 9–13, intron 13, and exon 14. Using WT htau as a template, two htau constructs termed AP or E14 were generated by mutating all 14 S/P or T/P amino acid residues (T111, T153, T175, T181, S199, S202, T205, T212, T217, T231, S235, S396, S404, and S422; numbering based on the longest 441-amino acid brain isoform of htau) to alanine (AP) or glutamate (E14). The AP/P301L or E14/P301L htau construct was generated by mutating the proline to leucine at residue 301 in AP or E14 htau, respectively. The PCR- mediated site-directed mutagenesis was confirmed by sequencing.

Concentrations used were: 10 μM morphine, 10μM naloxone, 10μM D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2 (CTOP), 10 μM KN-62 (Tokumitsu et al., 1990), 1 μM FK506 (Kam et al., 2010; Lieberman and Mody, 1994), 30μM N,N,N,-trimethyl-5-[(tricyclo[3.3.1.13,7]dec-1-ylmethyl) amino]-1-pentanaminiumbromide hydrobromide (IEM-1460) (Fortin et al., 2010) 3 μM

CHIR 99021 (CHIR). Drugs were added to the culture media at 21 DIV and remained for the length of the experiments.

Oligomerized $A\beta_{1-42}$ Preparation

Synthetic $A\beta_{1-42}$ was prepared as previously described (Stine et al., 2003) with some modification. $A\beta_{1-42}$ was a product of Sigma Aldrich Corporation (A9810 -0.1mg) and was purchased in 0.1 mg aliquots. $A\beta_{1-42}$ was suspended into 22.17 µL of 1,1,1,3,3,3 –Hexafluoro-2-Propanol (HFIP). After suspension, $A\beta_{1-42}$ was incubated at room temperature for 30 minutes and dried using a SpeedVac for one hour at 30°C. $A\beta_{1-42}$ was resuspended in DMSO to create a 5 mM concentration. Resuspended $A\beta_{1-42}$ was sonicated for 10 min. After sonication, F-12 solution was added to the $A\beta_{1-42}$-DMSO suspension to create a final concentration of 100 µM. $A\beta_{1-42}$ was incubated at 4° C for 14 days. After 14 days, a western blot was performed to verify oligomer existence within $A\beta_{1-42}$ mixture. Cells were treated with 2 µM oligomerized $A\beta_{1-42}$ in our experiments.

Western blots were used to confirm dimeric, trimeric, and high order species within oligomerized $A\beta_{1-42}$. Oligomerized $A\beta_{1-42}$ western blots were performed by Dr. Karen Ashe's lab as previously described by Lesne et al. (Lesne, et al., 2006). Oligomerized $A\beta_{1-42}$ samples were diluted 1:1000 in IP dilution buffer (IPDB). IPDB was made by adding 50 mL of 1M Tris-HCL and 8.76g NaCl to 1L of water. 50µL of protein G sepharose B Flat Flow beads were added to each sample. Suspensions were nutated for 1 hour 4°C and centrifuged at 9200g for 5 minutes at 4°C. Supernatants were collected and 5 µg of 6E10 antibodies (1:2500) were added to each sample and suspended overnight. Samples were washed using IP buffer A and IP buffer B. IP buffer A contained 50 mL of 1M Tris-HCL, a mL of Triton X-100, 17.52g NaCl, and 0.372g EDTA. IP buffer B contained IP buffer A contained 50 mL of 1M Tris-HCL, a mL of Triton X-

100, 8.76g NaCl, and 0.372g EDTA. Samples of oligomerized $A\beta_{1-42}$ were eluted using IPDB and loading buffer.

To run western blots, 2μg of oligomerized $A\beta_{1-42}$ were aliquoted and resuspended in tricine buffer and size fractioned by PAGE using pre-cast 10% SDS Tris-Tricine gels. Gels were blotted using nitrocellulose membranes were boiled twice in 50 mL PBS. Membranses were blocked in Tri-buffered saline 0.1% containing 5% bovine serum for 2 hours at room temperature and then probed with antibodies blocking buffer. Primary antibodies were detected with anti-IgG immunoglobulins conjugated with either biotin or horseradish peroxidase. Before cells were treated with oligomerized $A\beta_{1-42}$, samples were verified to ensure content of toxic oligomeric dimers and trimers.

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